

CHARACTERIZATION OF MOUSE ADENOVIRUS
AND OTHER
PREREQUISITE EXPERIMENTS
TO THE
DEFINITION OF THE ADENOVIRUS REPLICON

Inaugural-Dissertation
zur
Erlangung des Doktorgrades
des
Fachbereichs Chemie und Pharmazie
der
Ludwig-Maximilians-Universität
München

vorgelegt von
Margaret Pauline Temple

München 1980

CHARACTERIZATION OF MOUSE ADENOVIRUS
AND OTHER
PREREQUISITE EXPERIMENTS
TO THE
DEFINITION OF THE ADENOVIRUS REPLICON

INAUGURAL-DISSERTATION
ZUR
ERLANGUNG DES DOKTORGRADES
DES
FACHBEREICHES CHEMIE UND PHARMAZIE
DER
LUDWIG-MAXIMILIANS-UNIVERSITÄT
MÜNCHEN

VORGELEGT VON

MARGARET PAULINE TEMPLE

MÜNCHEN 1980

1. Berichterstatter: Prof.Dr.E.-L. Winnacker

2. Berichterstatter: Prof.Dr.G.Hartmann

Tag der mündlichen Prüfung: 23. Juli 1980



This work
is dedicated to

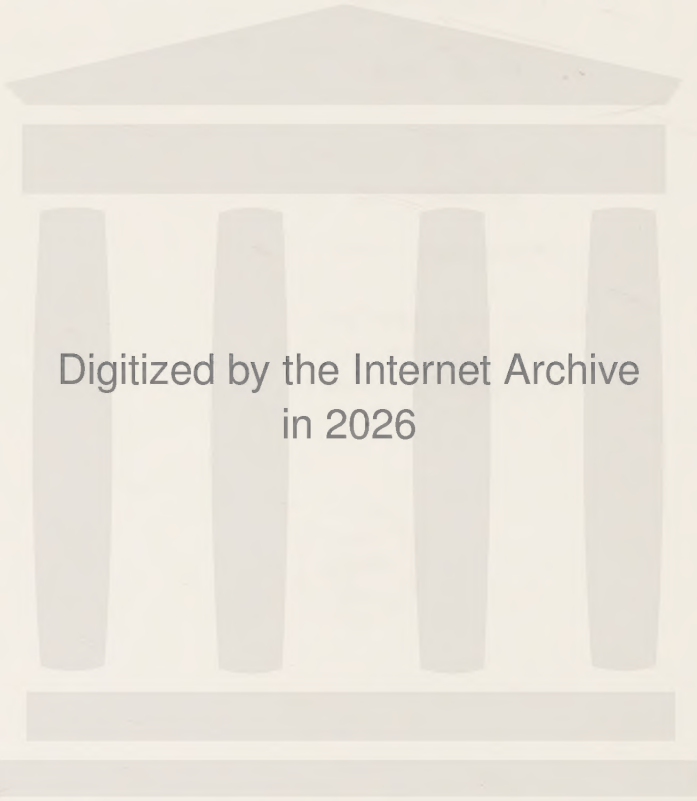
P.H.D. Lang

and

in memory of

Flora Bade Lang

who so very long ago
introduced me to
the Wonders of Nature



Digitized by the Internet Archive
in 2026

Die vorliegende Arbeit wurde von Juli 1977 bis Juli 1980 auf Anregung und unter Anleitung von Herrn Professor Dr. E.-L. Winnacker am Institut für Biochemie der Ludwig-Maximilians-Universität München ausgeführt.

I wish especially to thank Dr. G. Hartmann for inviting me to Munich in the first place and for advice, help and support during the time I have been here.

I wish to thank Dr. E.-L. Winnacker for inviting me to work in his laboratory, providing me with a project and the freedom to carry it out, and above all for advice and help when I most needed it.

I wish to thank Theresia Reiter for befriending me as I arrived a stranger in Munich.

I wish to thank Frau A. Winnacker and all others, who helped me to establish a household here.

I wish to thank Gary Rhodes for his ever continuing encouragement and support. Again without his help this work would neither have been started nor completed.

I wish to thank Gerhard Antoine for his comradeship in the struggle with Mad.

TABLE OF CONTENTS

page

INTRODUCTION

1

MATERIALS

1. Cells	8
2. Virus	8
3. Accessories, media and solutions for cell culture	8
4. Solutions for the isolation of virus	8
5. Solutions for the isolation of DNA-protein complex	8
6. Solutions for the isolation of DNA	9
7. Solutions for transfection	9
8. Solutions for electrophoresis	9
9. Solutions for sucrose gradient centrifugation	10
10. Radioactivity	10
11. Scintillation fluids	10
12. Supplies for autoradiography	10
13. Restriction enzymes	10
14. Stock solutions and other materials	11

METHODS

1. Cell growth	12
2. Growth of mouse adenovirus	12
a) On monolayers	12
b) In suspension culture	13
3. Isolation of mouse adenovirus	13
4. Growth and isolation of Ad 2 and Ad 5	14
5. Isolation of DNA	14
a) From virions	14
b) From infected cells	14
c) As DNA-protein complex	15
6. Transfection	16
7. Radioactive DNA	16
a) Pulse labeled for isolation from infected cells	16
b) Uniformly labeled for isolation from infected cells	16
c) Pulse labeled for analysis on sucrose gradients	16
d) Labeled at the 5' end	16

	page
8. Restriction enzyme digests	16
9. Gel electrophoresis	17
a) of protein	17
b) of DNA in polyacrylamide gel	17
c) of DNA in composite polyacrylamide-agarose gel	17
d) for molecular weight determination	17
e) for DNA strand separation	18
10. Sucrose gradient centrifugation	18

RESULTS

1. Growth of mouse adenovirus on cell lines	20
2. The virion proteins	21
3. The size of the DNA	22
4. The DNA has blocked 5' ends	25
5. Time and concentration kinetics of DNA synthesis in infected cells	27
6. Is the DNA synthesized after infection really mouse adenovirus DNA ?	30
7. Location of the termini of replication	32
8. How long does one round of replication take ?	33
9. Location of the origins of replication	34
10. Replication occurs via single-stranded intermediates	36
11. Transfection of HeLa cells with Ad 2 DNA-protein complex	37

<u>DISCUSSION</u>	41
-------------------	----

<u>REFERENCES</u>	50
-------------------	----

<u>SUMMARY</u>	63
----------------	----

<u>ABBREVIATIONS</u>	64
----------------------	----

<u>ZUSAMMENFASSUNG</u>	65
------------------------	----

<u>LEBENS LAUF</u>	66
--------------------	----

INTRODUCTION

This work concerns adenovirus, specifically the replication of its DNA during productive infection of tissue culture cells. There are other interactions between the virus and its host cell such as abortive infection, semipermissive infection and transformation (Green, 1970; Philipson and Lindberg, 1974; Ginsberg and Young, 1977) but I have only studied DNA synthesis leading to virus production.

After human adenoviruses were discovered (Rowe et al., 1953; Hilleman and Werner, 1954), classified (Rowe et al., 1958; Huebner, 1967), and named (Rowe et al., 1953; Enders et al., 1956) they quickly became objects of intensive biological and biochemical study. Human adenovirus serotype 2 (Ad 2) and serotype 5 (Ad 5) are the best characterized in terms of productive infection. Recently considerable information has been obtained concerning the mechanisms of transcription (Flint, 1977) and replication (Winnacker, 1978) of the linear, double-stranded DNA genome of these two types. In both DNA replication (Arens et al., 1977; Wold et al., 1978) and RNA synthesis (Weinman et al., 1974) the virus appears to exploit the host cell synthetic machinery to its own end of propagating itself. It thus is an attractive model for studying the macromolecular processes which occur in eukaryotic cells (Ginsberg and Young, 1977). Furthermore, DNA synthesis during productive infection is very efficient, making milligram quantities of viral DNA from 10^8 - 10^9 infected HeLa cells easily available (unpublished observations).

The essentials of a mechanism of DNA replication have been established, all that is except initiation (Winnacker, 1978). The DNA appears to replicate in a monomolecular, linear conformation with the origins and termini at both molecular ends (Winnacker, 1978).

The question of how a linear DNA molecule initiates and replicates to completion while at the same time providing the

necessary primer for the DNA synthesizing enzymes (Weissbach, 1977) is a general one for which several answering models have been proposed. Circularization of the DNA is one possibility as is found for the bacteriophage lambda (Young and Sinsheimer, 1967). The complex linear form is thus reduced to the simple circular form. Another possibility is that proposed by Watson (1972) in which linear molecules with appropriate terminally redundant sequences link themselves together end to end, forming long chains of molecules, or concatemers. Topographically this is the same as a circle because the majority of the molecules have no ends. Cavalier-Smith (1974) proposed still another model which allows for the complete replication of single linear molecules. Again the applicability depends on the primary structure of the molecular termini which must be palindromic and be able to form hairpin loops which serve as primers. Each of these schemes depends on a particular structure of the ends of the DNA in question.

Is any one of these models applicable to adenovirus DNA replication ?

Due to the structure of the DNA termini and lack of evidence for integration into the host cell genome as a required step in replication (Schick et al., 1976) application of any of these models is difficult to envisage. The problem is that the linear, uninterrupted, double-stranded DNA (Green et al., 1967) is not circularly permuted (Doerfler and Kleinschmidt, 1970; Murry and Green, 1973), does not have an inverted repetition as does the bacteriophage lambda (Strack and Kaiser, 1965), or a terminal redundancy as has T2 (MacHattie et al., 1967) and the other T_{even} bacteriophages, or that of the sort found for T3 and T7 (Ritchie et al., 1967). It does have an inverted terminal repetition (Garon et al., 1972; Wolfson and Dressler, 1972; Steenbergh et al., 1977; Arrand and Roberts, 1979; Tolun et al., 1979). These properties do not allow for the formation of double-stranded circles or concatemers.

Therefore, adenovirus is unable to employ the mechanisms proposed for initiation and termination at the ends of a DNA molecule. There must be another means of replicating a linear molecule to completion which we have not discovered yet.

One approach which seemed profitable in finding a solution to this problem was the isolation of the minimal adenovirus replicon, or the minimal amount of DNA required for replication, containing presumably only the origin and terminus and any needed control regions (Jacob et al., 1963). Similar approaches have been taken in defining the origin of SV40 DNA replication (Shenk, 1978; Subramanian and Shenk, 1978; Tjian, 1978) and in discovering a required region for plasmid Col E1 DNA replication (Backmann et al., 1978; Taylor and Cohen, 1979), bacteriophage lambda DNA replication (Hobom et al., 1978; Moore et al., 1978), and a host of others as described in the same volume. At very least studies of this type would serve to define a physical location for the origin which in connection with sequence data may provide clues to a mechanism of initiation. These studies I have started to do with mouse adenovirus.

In view of the abundance of information concerning the human adenovirus the reader may ask why it is necessary to study yet another adenovirus, especially one about which practically nothing is known. Unfortunately, although the human adenoviruses are well studied, well characterized and well defined they are unsuitable for some types of studies designed to investigate the expression of eukaryotic genes. They may not for example be used to carry foreign genetic material and are therefore useless as vehicles designed to isolate one particular gene and study its expression in the absence of other complicating functions. This approach has proved fruitful in the study of bacterial gene expression where isolated genes such as those controlling lactose metabolism in *E. coli* were reintroduced into bacterial cells using plasmids (Gilbert and Muller-Hill, 1967; Reznikoff et al., 1969), phage (Gilbert and

Muller-Hill, 1967) or a combination of both (Gilbert and Muller-Hill, 1967) in order to study their mode of action and control. The mouse adenovirus on the other hand may be used in studies requiring the use of a vector. The mouse has the added advantage that its genetics is quite well defined, making combined genetic and biochemical studies possible in principle to carry out (Ginsberg and Young, 1977), again in analogy with bacteria. Furthermore, since the most intensive studies of the biochemistry of adenoviruses have been carried out with the human adenoviruses (Philipson and Lindberg, 1974; Flint, 1977; Ginsberg and Young, 1977; Winnacker, 1978), thorough investigation of another species will serve to generalize the results so far obtained as being universal for adenoviruses.

In 1960 Hartley and Rowe isolated the first presumptive mouse adenovirus (M.Ad.) during attempts to establish the Friend mouse leukemia virus (Friend, 1957) in tissue culture (Hartley and Rowe, 1960). The criteria on which they classified the isolate as an adenovirus included the size of the particle as determined by filtration, lability to heat, stability to ether, appearance of the cytopathic effect, and immunological cross-reaction with sera from guinea pigs infected with human adenovirus types 1 and 5. They referred to the strain which they investigated as FL.

Later, another report of a mouse adenovirus appeared. Hashimoto et al. (1966) in the process of isolating virus strains from the feces of apparently healthy mice, isolated strain K 87 which appeared to be an adenovirus. Their criteria were similar to those of Hartley and Rowe (1960) but they also examined the morphology of the virus. Whether this virus is the same as that isolated by Hartley and Rowe (1960) is not clear. Hashimoto et al. (1966) only mentioned that anti-K 87 sera did not neutralize strain FL, and later investigators seem to have confirmed this observation (Van der Veen and Mes, 1974; Wigand et al., 1977).

The studies which followed with both virus strains involved experimental infection of mice in order to determine the nature of the disease produced as well as the tissue speci-

ficity of the virus. Sugiyama et al. (1967), using their isolated strain K 87, found that the virus appeared preferentially in the intestinal tract of mice of several age groups challenged with virus inoculated by any one of several routes. They found also that no condition of immunological tolerance could be induced. On the other hand Heck et al. (1972), studying the effects on mice and on tissue culture cells of infection with strain FL found a wider tissue range for the virus. It was for this reason that the current investigators of mouse adenovirus have chosen type FL (Larsen and Nathans, 1977).

Larsen and Nathans (1977) carried out the first characterization of the physical properties of the mouse adenovirus type FL virion. They too examined the morphology of the particle (Larsen and Nathans, 1977), but concentrated mainly on the properties of the DNA (Larsen and Nathans, 1977; Larsen et al., 1979). They determined the size of the DNA (Larsen and Nathans, 1977) and mapped the location of the cleavage sites of several restriction enzymes (Larsen and Nathans, 1977; Larsen et al., 1979). They presented indirect evidence for a protein bound to the ends of mouse adenovirus DNA and for a terminal repetition (Larsen and Nathans, 1977). Both of these properties are characteristics of the human adenoviruses. These studies combined quite clearly bestowed on mouse adenovirus the physical properties of an adenovirus; however, the question of the similarity of the molecular biological and biochemical properties still remained.

I have then undertaken investigations of the mouse adenovirus with the following goals in mind. First I wished to characterize the virus in terms of its physical and functional properties. This would at very least determine if mouse adenovirus is indeed an adenovirus, something which was not unambiguously clear from previous investigations (Hartley and Rowe, 1960; Larsen and Nathans, 1977; Wigand et al., 1977; Larsen et al., 1979). In this realm I have compared the physical properties of the virion, specifically the structure of its DNA and proteins, to those of the human adenoviruses. The results

compliment those which were recently reported by others (Larsen and Nathans, 1977; Antoine, 1979; Larsen et al., 1979). Most importantly I have characterized the growth and DNA replication of this virus and compared the results to those obtained with the well studied human adenoviruses.

Adenovirus DNA replication occurs with a mechanism having very well defined properties (Winnacker, 1978). Replication occurs via single-stranded intermediates (Lechner and Kelly, 1977) and starts and finishes each round of replication at the molecular ends of the DNA (Weingärtner et al., 1976). I wished to show that mouse adenovirus DNA replication also has these properties. Apparent identity of the replication schemes would provide generality for the model proposed on the basis of results obtained for the human adenoviruses.

A second goal which depends on the phenomenon of mouse and human adenovirus DNA replication being identical is the definition of the adenovirus replicon. Specifically this means determining the minimal amount of DNA which can still replicate in the presence of the appropriate helper functions. In this manner the origin of replication may be identified.

The functional aspects of any control region such as the origin of DNA replication are related to the physical structure (Subramanian et al., 1977). With the indication of the structural similarities between mouse adenovirus and the human adenoviruses (Hartley and Rowe, 1960; Hashimoto et al., 1966; Larsen and Nathans, 1977; Antoine, 1979; Larsen et al., 1979) as well as the functional similarities (Hartley and Rowe, 1960; this work) and with the advent of simple DNA sequencing technology (Maxam and Gilbert, 1977) it is possible to compare the presumed origin regions of adenoviruses with each other (Tolun et al., 1979) and with the origin regions of other viruses which infect the same host cell. Thus, on a purely intellectual level, similar structural features which may be relevant to functional identity may be determined. Such is the case for promotor sequences of prokaryotes (Pribnow, 1975; Schaller

et al., 1979), and transcriptional control sequences of eukaryotes (Soeda et al., 1980). Of course the actual relationship between the sequence (structure) and function must be determined experimentally.

A third goal is the development of a eukaryotic vector system exploiting the mouse adenovirus replicon. This at the moment is almost futuristic in prospect since neither I nor anyone else has progressed past the stage of initial characterization. There are also difficulties involved with introducing adenovirus DNA into cells.

Although the infection process is very efficient with every cell becoming infected at high multiplicities (Green, 1970) and although adenovirus DNA is infectious as determined by a plaque assay (Graham and Van der Eb, 1973), the transfection process is for some reason very inefficient. The reported infectivities range from 0.1 pfu/ μ g DNA (Nicolson and McAllister, 1972) to 5×10^5 pfu/ μ g DNA (Galos et al., 1979). For comparison the infectivity of virus, assuming 100% infectious particles, is greater than 10^{10} pfu/ μ g DNA! However, the higher reported values for infectivity of DNA do not really reflect the infectivity of 1.0 μ g DNA. They are instead calculated specific infectivities, that is, the infectivity observed with a very small amount of DNA normalized to 1.0 μ g DNA, assuming a linear relationship between plaque formation and the amount of DNA. Unfortunately, this linear relationship does not seem to be true. I have therefore returned to the well studied Ad 2 in order to better define the conditions required to make transfection an assay of the infectivity of the DNA.

MATERIALS

1. Cells

- a) HeLa were described by Weingärtner (1977).
- b) 3T3 (Balb/3T3 clone A31) were purchased from Flow Laboratories.
- c) L (LS) were purchased from Flow Laboratories.

2. Virus

- a) Human adenovirus serotype 2 was described by Weingärtner (1977).
- b) Human adenovirus serotype 5 was obtained from Ch.Meinschad.
- c) Mouse adenovirus type FL (Hartley and Rowe, 1960) was provided by Dr. D. Nathans. This virus had been plaque purified from virus obtained from Microbiological Associates, Bethesda, Maryland (Larsen and Nathans, 1977). The virus used in the studies I discuss here was grown from a single plaque (plaque A) obtained after plating the original stock on 3T3 cells.

3. Accessories, media and solutions for cell culture

The media and solutions used for cell culture and virus growth were described by Weingärtner (1977). They were prepared by P.Graner, J.Kummer and I.Pfitzinger. Plastic culture flasks and dishes were purchased from Nunc.

4. Solutions for the isolation of virus

The materials and solutions were described by Weingärtner (1977) with the addition of CsCl (Baker) solutions in 0.02 M Tris Cl pH 8.7 of densities 1.2, 1.34 and 1.5 g/cc.

5. Solutions for the isolation of DNA-protein complex

These were described by Fütterer (1978).

6. Solutions for the isolation of DNA

VD buffer (10 mM Tris Cl pH 8.0, 250 mM NaCl, 10 mM MgCl_2 , 1 mM EDTA)

TE buffer (10 mM Tris Cl pH 8.0, 1 mM EDTA)

SSC buffer (150 mM NaCl, 15 mM NaCitrate)

DNA buffer (20 mM Tris Cl pH 7.4, 50 mM NaCl, 1 mM EDTA)

Lysis buffer (20 mM Tris Cl pH 8.0, 5 mM EDTA, 0.6% SDS)

TEN buffer (10 mM Tris Cl pH 7.4, 5 mM EDTA, 100 mM NaCl)

B+D buffer (10 mM Tris Cl pH 8.0, 5 mM EDTA, 250 mM NaCl)

7. Solutions for transfection

The materials and solutions were described by Ullrich (1978) with the addition of 20% glycerol (Baker) in Tris-saline as described by Galos et al. (1979). K.Ullrich provided these materials.

8. Solutions for electrophoresis

The materials and solutions for electrophoresis were described by Weingärtner (1977) with the addition of TEB buffer (90 mM Tris base, 2.5 mM EDTA, 90 mM boric acid)

TEA buffer (40 mM Tris acetate pH 7.9, 1 mM EDTA, 20 mM NaAcetate)

PET buffer (30 mM Tris base, 36 mM NaH_2PO_4 , 1 mM EDTA)

stop buffer (10 mM Tris Cl pH 8.0, 50 mM EDTA, 1% SDS, 0.05% bromphenol blue, 0.025% xylene cyanol, 30% glycerol)
30% w/v acrylamide (Serva)

1% w/v bisacrylamide (Serva)

running buffer (3 g/l Tris base, 14.4 g/l glycine, 1 g/l SDS)

sample buffer (0.1 M DTT (Roth), 2% SDS, 0.08 M Tris Cl pH 6.8, 10% glycerol, 0.0012% bromphenol blue)

staining solution (0.25% commassie blue, 7.5% acetic acid, 10% methanol)

destaining solution (7.5% acetic acid, 10% methanol)

9. Solutions for sucrose gradient centrifugation

5, 20, 70% sucrose in 0.3 M NaOH, 0.7 M NaCl, 5 mM EDTA (alkaline sucrose)

5, 20, 70% sucrose in 10 mM Tris Cl pH 8.0, 1 M NaCl, 5 mM EDTA, 1% sarkosyl (Serva) (neutral sucrose)

heparin (Sigma) 1 mg/ml in 10 mM Tris Cl pH 8.0, 1 mM EDTA, 1% sarkosyl

alkaline buffer (0.9 M NaOH, 0.6 M NaCl, 20 mM EDTA)

neutral buffer (10 mM Tris Cl pH 8.0, 0.6 M NaCl, 20 mM EDTA, 1% sarkosyl)

10. Radioactivity

^3H -thymidine (20 Ci/mmol; Amersham Buchler)

^{32}P (HCl free; New England Nuclear)

γ - ^{32}P -ATP (New England Nuclear)

11. Scintillation fluids

These were described by Antoine (1979) with the addition of T+M (55 g Permablend III (Packard) in 5 liters toluene and 5 liters methanol).

12. Supplies for autoradiography

These were described by Antoine (1979).

13. Restriction enzymes

These were isolated by members of the laboratory of E.-L. Winnacker or purchased from Boehringer.

14. Stock solutions and other materials

The following buffers were prepared as 10-fold concentrated stock solutions and diluted before use: VD, SSC, DNA, TEN, B+D, TEB, TEA, PET.

All buffers and solutions were prepared with double-distilled water.

Dialysis tubing was boiled two times for 10 minutes in 5% NaCarbonate, 1 mM EDTA and then two times in 5 mM EDTA. It was stored in 5% ethanol, 1 mM EDTA at 4°. Chemicals were purchased from Merck unless otherwise indicated.

METHODS

1. Cell growth

The methods were described by Weingärtner (1977).

2. Growth of mouse adenovirus

a) On monolayers.

Just confluent or almost confluent 3T3 cells growing in monolayers were washed with 1/2 tsd (a mixture of one part Dulbeccos medium and one part Tris-saline). The virus in Dulbeccos or diluted appropriately with 1/2 tsd was added in 5% of the volume of medium later to be added for growth. Medium consisting of Dulbeccos supplemented with 2% new born calf serum was then added. Incubation was carried out at 37°.

For the production of virus and for making virus stocks, cells grown in 264 ml culture flasks were infected with a multiplicity of approximately 10 pfu/cell and incubation carried out for 3-4 days when a cytopathic effect became apparent (Hartley and Rowe, 1960). The growth medium was then discarded and replaced with 20 ml of Dulbeccos if the virus was to be used as stocks or with 2 ml if the virus was to be isolated. The flasks were frozen and the lysate resulting after thawing was used directly as stock virus or for virus isolation.

For determination of virus titers 3T3 monolayers growing on 35 mm or 60 mm plastic culture plates were infected with appropriate dilutions of virus as described except that after the adsorption period of one hour at 37°, an overlay consisting of Dulbeccos, 5% fetal calf serum, and 0.8% agarose (Sigma) was applied instead of growth medium. Incubation was carried out at 37° for 7 days after which time an overlay of 1/3 the volume of the original overlay and of the same composition except for the addition of 0.02% neutral red (Serva) was applied. After 12-14 hours the plaques were counted.

b) In suspension culture

L cells were pelleted from the culture by centrifugation at 1200 rpm for 10 minutes. They were then suspended in 5% of the original volume of Spinner medium and the virus was added. The suspension was stirred for 1 hour at 37° and afterwards medium consisting of Spinner supplemented with 2% fetal calf serum was added to the original volume. Incubation was carried out with stirring for 7 days at 37°. The cells were then pelleted as above and suspended in 2 ml of 0.02 M Tris Cl pH 8.0 and frozen. E.-L.Winnacker carried out these manipulations.

3. Isolation of mouse adenovirus

Virions were isolated from infected cultures basically as described by Larsen and Nathans (1977). Approximately 100 ml of the lysate resulting from freezing and thawing the infected cells was sonicated for 30 seconds with the large tip of a Branson sonifier (model B-12A) set to maximum output. When needed the lysate was titrated before sonication back to a bright pink color with 1 M Tris base. The sonicate was then centrifuged for 10 minutes at 3000 rpm in a Sorvall SS34 rotor to remove the cell debris. The sonicate was then layered into steps of CsCl consisting of 3 ml of 1.5 g/cc under 5 ml of 1.2 g/cc in DuPont/Sorvall O3141 tubes. The relative basicity of the CsCl solutions was necessary, due to the acidity of the 3T3 cells, and proved to improve the stability and thus the yield of virus. The gradients were centrifuged for 90 minutes at 24,000 rpm in a DuPont/Sorvall AH627 or a Beckmann SW 27 swinging bucket rotor. The resulting band(s) which appeared slightly below the interface between the CsCl and the medium were collected by removing and discarding the bottom-most 2-4 ml of CsCl and then removing the virus band in the next 1-2 ml. All operations were performed with a pasteur pipette inserted through the gradient from the top.

Centrifugation to equilibrium followed in DuPont/Sorvall O3127 tubes in a DuPont/Sorvall vertical rotor. This required

at least 3 hours at 47,000 rpm. The resulting band (the bottom-most in the case of multiple bands) was removed in the manner described above and centrifuged once or twice more to equilibrium. In the case of the equilibrium centrifugations CsCl of density 1.34 g/cc was added when extra volume was required. This is the density of the virus in CsCl (Larsen and Nathans, 1977). The resulting band was removed and dialyzed at least 6 hours against 1000 volumes of VD buffer. All of the procedures described above were carried out at 4° or with the virus on ice.

4. Growth and isolation of Ad 2 and Ad 5

The methods were described by Weingärtner (1977). P.Graner, I.Pfützinger, K.Ullrich and E.-L. Winnacker provided the Ad 2. Ch.Meinschad provided the Ad 5.

5. Isolation of DNA

a) From virions.

Dialyzed virions were incubated at 37° for two hours in the presence of 1% SDS and 100 µg/ml proteinase K. Afterwards the protein was removed by extraction with redistilled phenol which was saturated with TE buffer. The DNA was then dialyzed against at least two changes each of 1000 volumes of SSC in order to remove the phenol and then into DNA buffer. To test for the absence of phenol the absorption spectrum of the DNA from 230-290 nm was measured. The concentration of the DNA was calculated from the absorption at 260 nm by use of the extinction coefficient $E_{260}^{1 \text{ mg/ml}} = 20$ (Van der Eb et al., 1969).

b) From infected cells.

Viral DNA was isolated from infected cells utilizing a modified (Schilling et al., 1975) Hirt procedure (Hirt, 1967). Medium from infected cells growing on 100 mm plastic culture plates was discarded and the cells were washed twice with 1/2 tsd and with PBSd once. The cells were removed from the plates after the addition of 1 ml of lysis buffer to each plate. They were incubated in the presence of 100 µg/ml

proteinase K for 2 hours at 37°. Afterwards 5 M NaCl was added to result in a final concentration of 1 M. Incubation at 4° followed for at least 4 hours. The precipitated cellular DNA was pelleted by centrifugation at 25,000 rpm for 35 minutes in either a DuPont/Sorvall AH 627 or AH 650 swinging bucket rotor depending on the volume of the lysate. The supernatant was dialyzed against 2 changes of approximately 50-100 volumes of TEN. Protein was removed by extraction with an equal volume of redistilled phenol which was saturated with TEN. Dialysis followed against multiple changes of SSC to remove the phenol and then against B+D. Separation of completed double-stranded molecules from incomplete molecules with single-stranded regions was accomplished by chromatography on BND cellulose (Schilling et al., 1975). Double-stranded molecules were eluted with (B+D)+1 M NaCl and single-stranded molecules were eluted with (B+D)+1 M NaCl + 2% caffeine. The DNA was precipitated by adding 1.5 volumes of isopropanol in the presence of 0.3 M NaAcetate, incubating the mixture at -20° for at least 12 hours, and centrifugating at 4° for several hours at 9,000 - 12,000 rpm in a Sorvall SS34 rotor. The precipitated DNA was suspended in an appropriate volume of DNA buffer and then dialyzed against the same buffer to remove the isopropanol and the salt.

c) as DNA-protein complex.

The method was described by Fütterer (1978).

6. Transfection

Transfection of HeLa cell monolayers with Ad 2 DNA protein complex was carried out using the Ca^{+2} precipitation technique of Graham and Van der Eb (1973) with the DMSO treatment as described by Stow and Wilkie (1976), or glycerol treatment as described by Frost and Williams (1978). The methods were described by Ullrich (1978).

7. Radioactive DNA

- a) Pulse labeled for isolation from infected cells.

The growth medium was discarded from infected cells growing on 100 mm plates, 50 μ Ci of ^3H -thymidine in 1 ml Dulbeccos was added to each plate and incubation was carried out for the appropriate length of time at 37° .

- b) Uniformly labeled for isolation from infected cells.

50 μ Ci of ^3H -thymidine in 1 ml Dulbeccos was added directly to the growth medium of cells growing on 100 mm plates and incubation continued for at least 12 hours at 37° .

- c) Pulse labeled for analysis on sucrose gradients.

10 μ Ci of ^3H -thymidine per milliliter of growth medium was added and incubation continued for 2 hours at 37° .

- d) Labeled at the 5' end.

Labeling the 5' end of DNA or restriction fragments was carried out according to the method of Maxam and Gilbert (1977). This was the forward reaction of T4 polynucleotide kinase under conditions for DNA with protruding 5' ends (Maxam and Gilbert, personal communication). Synthesis of $\gamma\text{-}^{32}\text{P}$ -ATP and treatment of the DNA with bovine intestinal alkaline phosphatase was carried out as described by Antoine (1979).

8. Restriction enzyme digests

DNA in DNA buffer was diluted as needed with DNA buffer. MgCl_2 was added to a concentration of 10 mM and enzyme was added, usually 5-10% of the reaction volume. Incubation was carried out at 37° . Either the reaction was terminated by the addition of stop buffer or further steps were carried out.

9. Gel electrophoresis

a) of protein.

Analysis of virion protein was carried out in 10% polyacrylamide slab gels of the composition described by Laemmli (1970). The samples were prepared by dissolving dialyzed virions in 1% SDS, adding an equal volume of sample buffer and heating the samples for 2 minutes at 100° before loading them on the gel. Electrophoresis was carried out at 130 V in running buffer until the bromphenol blue marker was 1 cm from the bottom of the gel. After electrophoresis the gels were stained with staining solution which was diluted 5-fold with destaining solution, and then destained.

b) of DNA in polyacrylamide gel.

The 5% gels were described by Maxam and Gilbert (1977) for "strand separation".

c) of DNA in composite polyacrylamide-agarose gel.

Conditions for electrophoresis of DNA in composite polyacrylamide-agarose gel have been described as has the procedure for processing the gels into 1 mm slices for liquid scintillation counting (Weingärtner et al., 1976). Protein was not removed from the samples after restriction enzyme treatment. They were instead applied to the gels immediately after addition of stop buffer. The electrophoresis buffer was TEB containing 0.5% sarkosyl. The counting data were recorded on paper tape. They were analyzed and plotted courtesy of the Ges.f.wiss.Datenverarbeitung mbH, Göttingen, with a program written by G.Rhodes.

d) for molecular weight determination.

Electrophoresis of DNA in low percentage agarose gel in order to determine the molecular weight was carried out according to Fangman (1978) in 0.2% horizontal agarose slab gels.

e) for DNA strand separation.

Electrophoresis in order to separate the strands of DNA restriction fragments was carried out basically according to the procedure of Perlman and Huberman (1977). Restriction fragments were separated by electrophoresis though 1% agarose (1.5 cm diameter tube gels) in TEA buffer. The appropriate fragments were cut out after they had been stained with ethidium bromide and the gel pieces containing the fragments were soaked for 1 hour at room temperature in 0.3 M NaOH. Afterwards they were transferred to ice-cold PET buffer and soaked for 10 minutes. The pieces were then fused onto 1.4% agarose (1.5 cm diameter tube gels) in PET buffer. Electrophoresis was carried out at room temperature for 20 hours at 2-5 V/cm. The appropriate bands were cut out after the gel was stained again with ethidium bromide. These were melted in 1 ml of water and immediately 20 ml of T+M was added and the samples counted. The counting efficiency was 5-10%.

10. Sucrose gradient centrifugation

Centrifugation through alkaline and neutral sucrose was carried out in 4.6 ml DuPont/Sorvall 03127 tubes, basically as described by Winnacker (1975) and Kathmann et al. (1976). The gradients consisted of a 0.2 ml cushion of 70% sucrose and a 4 ml linear gradient of 5-20% sucrose. Samples which consisted of an appropriate number of counts from pulse labeled infected cells which had been scraped off the plates and suspended in a small volume of 1/2tsd were layered on the gradients along with an equal volume of heparin solution. After 20 minutes at room temperature (Walters and Hildebrand, 1975) twice the sample volume of alkaline or neutral buffer was added and lysis of the cells allowed to occur for 8-12 hours at 4⁰. Centrifugation followed, also at 4⁰, for 1.5 - 2 hours at 47,000 rpm in a DuPont/Sorvall AH650 swinging-bucket rotor. Fractions of 3 drops in the case of alkaline gradients and 5 drops in the case of neutral gradients were collected by inserting a 20 µl glass micropipette through the gradient from the top and pumping the solution from the bottom directly

into scintillation vials for the addition of 10 ml of T+M. The counting data were recorded on paper tape and analyzed as described for the gel electrophoresis data.

RESULTS

1. Growth of mouse adenovirus on cell lines.

I first determined the yields of virus grown on 3T3 cell monolayers and on L cells growing in suspension culture in order to obtain high titer virus stocks and to determine the best cell line to use in the subsequent biochemical studies. Table 1 shows the yields of virus grown on these two

Table 1. Yields of mouse adenovirus grown on 3T3 and L cells.

Virus stock	host cell	growth time	m.o.i. (pfu/cell)	yield (pfu/cell)
1 ^a	3T3	8 days	-	100
2 ^b	3T3	100 hours	3	100
3 ^b	L	7 days	2×10^{-2}	10^{-3}
4 ^b	L	7 days	2×10^{-2}	10^{-5}

a = from infection with plaque A

b = from infection with stock 1

cell lines. The amount of virus produced per cell is consistantly higher for virus grown on 3T3 monolayers as was also observed by Larsen and Nathans (1977). The reason for this difference is not known although there are several possible explanations at least for the results presented here.

One possibility is that the medium used for the suspension cultures lacks Ca^{+2} , folic acid and pyruvate, and the concentration of several other components is different than in the medium used in monolayer cultures. It is possible that the virus requires one or more of these substances as a cofactor. Several bacteriophages and viruses need certain metal ions or amino acids in order to infect their host cells or to complete the infection process. Bacteriophages T1 and T5 require Ca^{+2} (Puck, 1949; Luria and Steiner, 1954). Certain mutants of T4 require

tryptophan (Anderson, 1948), and adenovirus itself requires arginine for optimal yield (Rouse and Schlesinger, 1967; Russell and Becker, 1968). Another possibility is that the length of time allowed for growth of the virus on the L cells was not long enough for the very low multiplicity of infection. A third possibility is that the L cells are somehow intrinsically deficient in some component or function necessary for virus production. In any case I did not investigate this problem further but rather chose to grow the virus routinely on 3T3 monolayers.

The average yield of virus of 100 pfu per cell is 10-fold lower than that reported by Wigand et al. (1977) for the growth of the virus on primary cultures of mouse kidney or mouse embryo cells. However, it is about the same as that reported by Larsen and Nathans (1977). For comparison the yield of Ad 2 grown on HeLa cells under similar conditions approaches 10^4 pfu per cell (unpublished observations). The virus appears to grow with the highest yield in plastic culture flasks or dishes. Growth in glass bottles, glass roller bottles and plastic roller bottles was highly unsatisfactory on the basis of the amount of virus obtained after isolation from lysates of infected cells grown in these containers.

2. The virion proteins.

On the basis of what was seen in electronmicrographs of intact virus particles mouse adenovirus appeared to be morphologically similar if not identical to the human adenoviruses (Hartley and Rowe, 1960; Larsen and Nathans, 1977). It should, therefore, also be constructed similarly. Shown in Figure 1 is an SDS-polyacrylamide gel (Laemmli, 1970) of the virions of Ad 2 and mouse adenovirus. Clearly seen is the characteristic protein pattern of Ad 2 (Maizel et al., 1968), at least for the larger proteins. The smaller proteins, with molecular weights below 20 K daltons have run off this gel. The mouse adenovirus proteins appear quite different. Although not much can be said

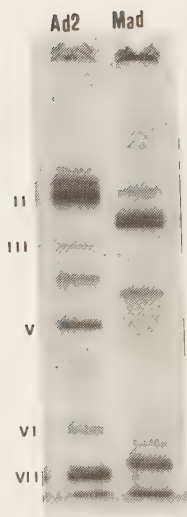


Fig. 1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of Ad 2 and mouse adenovirus particles. Preparation of the samples and electrophoresis was carried out exactly as described in the methods section. Varying amounts of sample were applied until the protein bands were optimally visible. Ad 2 is adenovirus serotype 2. The major Ad 2 proteins appearing on this gel are indicated according to Maizel et al. (1968). Mad is mouse adenovirus.

about these results the mouse adenovirus hexon is most likely the heavy band between Ad 2 protein II (hexon) and III, making it smaller than the Ad 2 hexon which has a subunit molecular weight of 120 K daltons (Grütter and Franklin, 1974).

3. The size of the DNA.

Ad 2 DNA has a molecular weight of 21.8×10^6 daltons (Doerfler and Kleinschmidt, 1970) and this seems to be the size of the DNA from most adenoviruses (Green et al., 1967). The avian adenovirus, CELO, has DNA which is slightly larger with a molecular weight of 29×10^6 daltons (Bellett and Younghusband, 1972). Therefore, I determined the molecular weight of mouse adenovirus

DNA isolated from virions by comparing its electrophoretic mobility in low percentage agarose gel to that of several DNA species of known molecular weight. A photograph of such a gel is shown in Figure 2. Gels of this type must be run with very

1 2 3 4 5 6 7 8

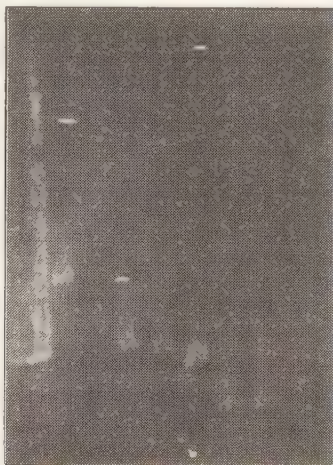


Fig. 2. Molecular weight determination of mouse adenovirus DNA by electrophoresis through 0.2% agarose gel. Approximately 0.012 μ g of DNA per sample was applied to a chilled (4°) horizontal 0.2% agarose gel as described by Fangman (1978). Electrophoresis was carried out for 20 hours at 80 V. The gel was then stained for one hour with 1 μ g/ml ethidium bromide in electrophoresis buffer (TEB) and photographed for 2 hours under illumination with UV light (366 nm). Position 1: T4 DNA, 2: Lambda DNA, 3: T7 DNA, 4: Ad 2 DNA, 5: Mouse adenovirus DNA, 6: Ad 2 Eco R1 A fragment, 7: Lambda Eco R1 A fragment, 8: Mouse adenovirus Eco R1 A fragment. The smaller fragments present in samples 6, 7 and 8 are no longer visible on this gel.

small amounts of DNA (Fangman, 1978). Already the 0.012 μ g of DNA per sample on this gel represents a slight overloading as indicated by the trailing bands. The sharp fronts were used as the reference point in the subsequent molecular weight analysis.

From a representation of the logarithm of the molecular weight as a function of the electrophoretic mobility (Figure 3) mouse adenovirus DNA has a molecular weight of $18-19 \times 10^6$ daltons.

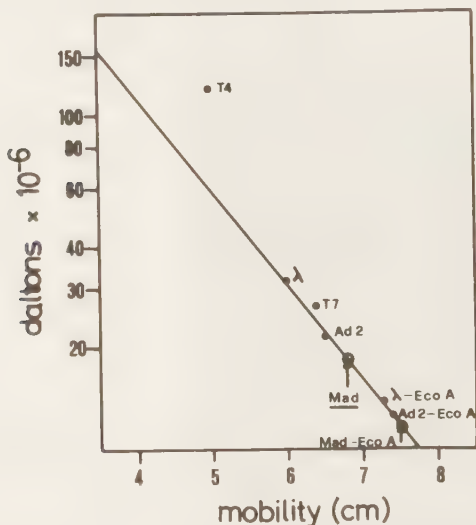


Fig. 3. Estimation of the molecular weight of mouse adenovirus DNA.

The logarithm of the molecular weights of the marker DNA molecules was plotted as a function of the electrophoretic mobility as measured from the gel shown in Figure 2. The position of the sharp fronts of the various bands was used as the distance that the molecules had migrated. The following values for the molecular weights were applied: T4 DNA = 105×10^6 daltons (Davidson and Szybalski, 1971), Lambda DNA = 31×10^6 daltons (Davidson and Szybalski, 1971), T7 DNA = 25×10^6 daltons (Davidson and Szybalski, 1971), Ad 2 DNA = 21.8×10^6 daltons (Doerfler and Kleinschmidt, 1970), Lambda Eco R1 A fragment = 13.7×10^6 daltons (Allet et al., 1973), Ad 2 Eco R1 A fragment = 12.9×10^6 daltons (Pettersson et al., 1973).

Larsen and Nathans (1977) measured the length of double stranded mouse adenovirus DNA in electronmicrographs and compared it to form II SV40 DNA. They arrived at a value of 20×10^6 daltons for its molecular weight. However, when they determined the length electrophoretically, or averaged all of the electron microscopic

measurements, the value they obtained was 19.5×10^6 daltons. They ascribed this discrepancy to the presence of broken or smaller molecules which occur naturally in the total population. Using denatured, single-stranded mouse adenovirus DNA circles and a relaxed PM 2 DNA standard, H. Delius (personal communication) measured a value of 20.7×10^6 daltons. All of these results agree well with each other and show that the DNA of mouse adenovirus is smaller than that of the human adenoviruses (Green et al., 1967) and of CELO (Bellett and Younghusband, 1972).

4. The DNA has blocked 5' ends.

Larsen and Nathans (1977) presented evidence for a protein bound to the ends of mouse adenovirus DNA. They observed reduced electrophoretic mobility of terminal restriction fragments from DNA which had been prepared under conditions which preserve the terminal protein of adenoviruses (Robinson and Bellett, 1974). Subsequent proteinase treatment of these retarded fragments eliminated this phenomenon. Although these observations present strong evidence for a protein it says nothing about its location at the ends of the DNA, specifically if it is bound to the 5' end as is the case for Ad 2 (Carusi, 1977; Rekosh et al., 1977). One of the lines of evidence which led to the conclusion that the terminal protein of Ad 2 is bound at the 5' end came from Carusi (1977) who observed that intact Ad 2 DNA could not be labeled with ^{32}P in the reaction with T4 polynucleotide kinase. Since the phosphate in that reaction is added to the 5' ends (Richardson, 1965) of nucleic acids this indicated that somehow the 5' end was blocked. The conclusion was that even in the case of DNA treated with proteolytic enzymes, a few amino acid residues still remained to block the 5' end and that therefore the protein was bound in a covalent linkage at that terminus.

When I performed this same experiment with intact mouse adenovirus DNA, the DNA was not labeled as seen in the autoradiogram

shown in Figure 4. In contrast the Eco R1 fragments of mouse adenovirus DNA which have free 5' ends were labeled. From this result I can conclude that the terminal protein of mouse adenovirus is bound at the 5' end of the DNA.



Fig. 4. Demonstration that the 5' ends of mouse adenovirus DNA are resistant to labeling in the reaction with T4 polynucleotide kinase. Mouse adenovirus DNA which had been isolated from virions by treatment with proteinase K and phenol was treated with phosphatase and then with T4 polynucleotide kinase. The samples were analyzed by autoradiography after electrophoresis through 5% polyacrylamide gel. Each sample contained 4 μ g of DNA. As a control of the enzymatic reactions the DNA of one sample was cleaved with Eco R1 to produce fragments with free 5' ends. Sample 1 is intact mouse adenovirus DNA and sample 2 is mouse adenovirus DNA treated with Eco R1. A, B and C indicate the position of the fragments, and Mad the position of intact DNA.

5. Time and concentration kinetics of DNA synthesis in infected cells

I analyzed the kinetics of viral DNA synthesis in infected cells both as a function of time and as a function of the multiplicity of infection in order to define the conditions under which DNA was most easily available. To determine the extent of viral DNA synthesis I lysed the infected cells on alkaline or neutral sucrose gradients and analysed the size distribution of the newly synthesized DNA. Profiles of typical alkaline gradients are shown in Figure 5, profiles of typical neutral gradients in Figure 6, and a summary of the data is presented in Table 2.

The alkaline gradients of Figure 5 show synthesis resulting from an infection of 0.3 pfu per cell, a low multiplicity of infection. Synthesis is not apparent until 60 hours after infection and is still continuing after 71 hours. Unfortunately the ^{32}P -labeled Ad 2 DNA used as marker in this experiment was very badly degraded and its position in the gradient does not accurately reflect the sedimentation of the newly synthesized mouse adenovirus DNA. In fact, in an attempt to adjust the position of the marker to be in the middle of the gradient, the time of centrifugation was much too long causing the newly synthesized DNA to be practically at the bottom of the gradient. That the material is actually viral sized DNA and not larger DNA is clear because there is no labeled material right at the tube bottom as is found at earlier times after infection. This lack of incorporation into rapidly sedimenting material once viral DNA synthesis is measurable is also evidence that the host cell DNA synthesis is shut off as is found for productive infection with the human adenoviruses (Pina and Green, 1969).

The neutral gradients shown in Figure 6 illustrate the kinetics of synthesis after infection with 3 pfu per cell, a relatively high multiplicity of infection. Here viral DNA synthesis becomes apparent after only 36 hours. In this case also there is no longer any rapidly sedimenting material synthesized once viral DNA

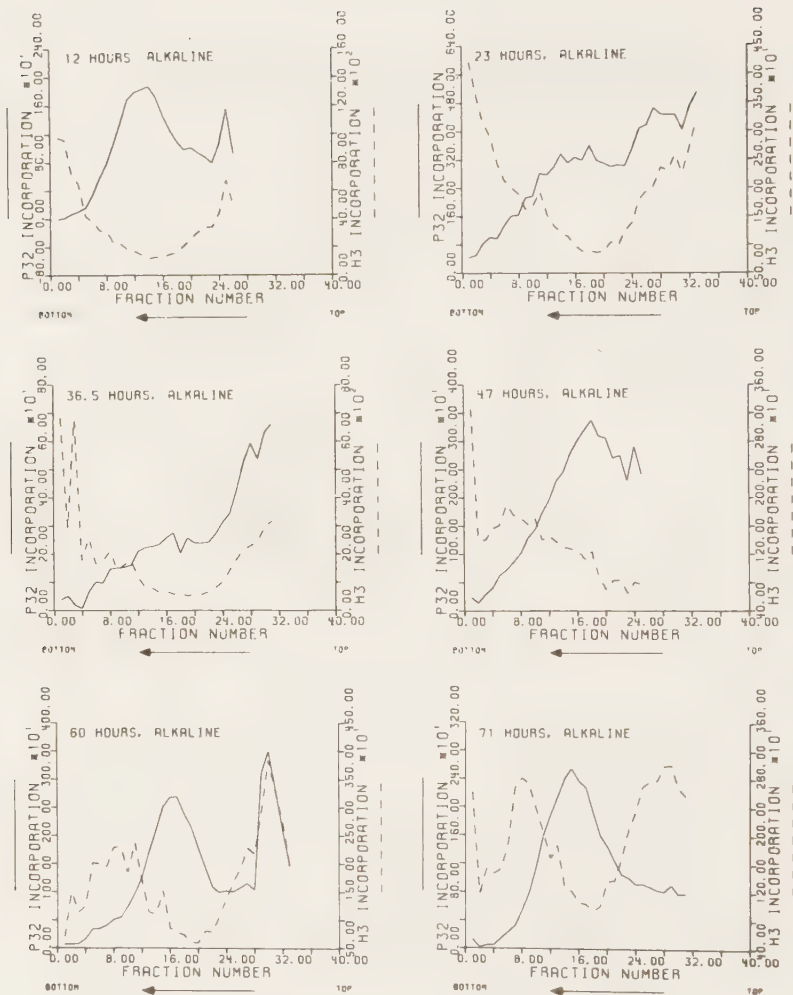


Fig. 5. Alkaline sucrose gradients of mouse adenovirus DNA synthesized at various times after infection. Subconfluent monolayers of 3T3 cells were infected at a multiplicity of 0.3 pfu/cell. Cells were labeled for two hours with H^3 -thymidine (10 μ Ci/ml) starting 12, 23, 36.5, 47, 60 and 71 hours after infection. The medium was then discarded and the cells were washed with 1/2 tsd. The cells from a 35 mm petri dish were scraped off with a rubber policeman and suspended in 0.5 ml of 1/2 tsd. Appropriate (in terms of the number of counts) aliquots were analyzed by sedimentation through alkaline sucrose gradients in the presence of P^{32} -labeled Ad 2 DNA as marker.

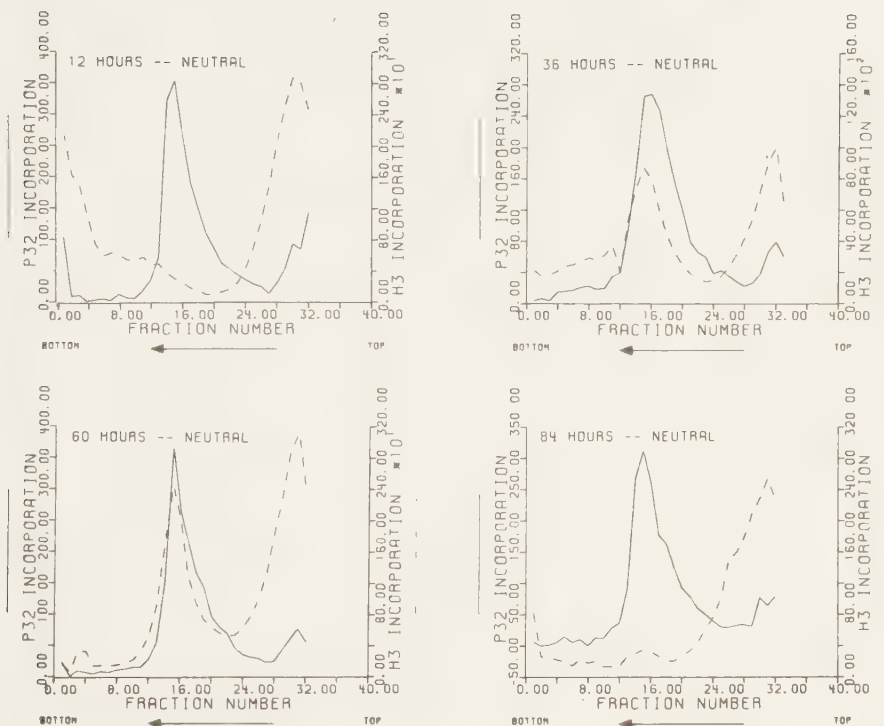


Fig.6. Neutral sucrose gradients of mouse adenovirus DNA synthesized at various times after infection. Subconfluent monolayers of 3T3 cells were infected at a multiplicity₃ of 3 pfu/cell. Cells were labeled for 2 hours with ^3H -thymidine (10 $\mu\text{Ci/ml}$) starting 12, 36, 60 and 84 hours after infection. The medium was then discarded and the cells were washed with 1/2 tsd. The cells from a 35 mm petri dish were scraped off with a rubber policeman and suspended in 0.5 ml of 1/2 tsd. Appropriate (in terms of the number of counts) aliquots were analyzed by sedimentation though neutral sucrose gradients in the presence of ^{32}P -labeled Ad 2 DNA as marker.

synthesis is measurable. By 84 hours there is no longer any synthesis occurring at all and the labeled material remains at the top of the gradient. These two examples are incorporated into Table 2 which shows that the time at which viral DNA synthesis begins to be measurable depends on the multiplicity of infection. With even lower multiplicities than 0.3 pfu per cell synthesis may not occur until 100 hours after infection. The minimum time seems to be about 36 hours; with multiplicities as high as 800 pfu per cell measurable synthesis occurs no earlier.

Table 2. Time and concentration kinetics of DNA synthesis in infected cells.

m.o.i.	hours p.i. neutral	hours p.i. alkaline
0.3	60	60
0.8	-	47
1.4	47	47
3.0	36	-
14	38	38

6. Is the DNA synthesized after infection really mouse adenovirus DNA ?

The DNA synthesized after infection sediments as a single species with a size consistent with its being mouse adenovirus DNA. However, I wished to show more conclusively that it is of viral origin. To do this I took advantage of the restriction maps for mouse adenovirus DNA which were published by Larsen and Nathans (1977). If the DNA found in infected cells is really mouse adenovirus DNA, then upon incubation with restriction enzymes it should be cleaved into the same fragments as DNA isolated from virions. Therefore I labeled infected cells 59-70 hours after infection, isolated the completed, double-stranded molecules, treated them with the restriction endonucleases Eco RI, Bam HI, Bgl II, Hpa I and Sal I, and separated the fragments on composite agarose-acrylamide gels. The results are shown in Figure 7. The patterns I observe for DNA isolated from infected cells are identical to those observed by Larsen and Nathans (1977) for virion DNA. I thus

concluded that the material synthesized after infection is indeed genuine mouse adenovirus DNA.

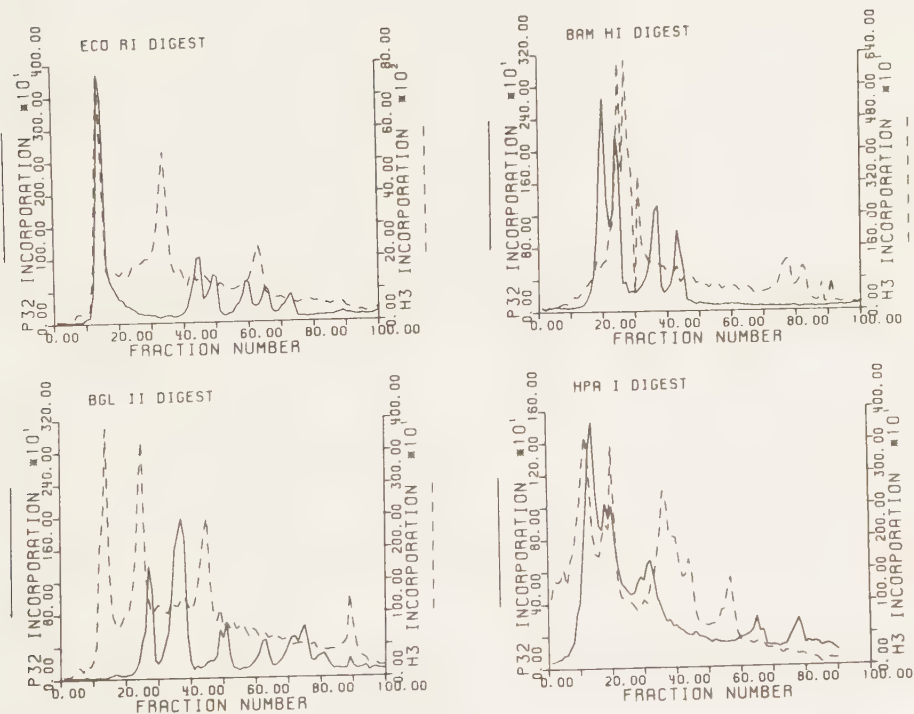


Fig.7. Gel electrophoresis of restriction digests of mouse adenovirus DNA isolated from infected cells. Viral DNA was isolated from infected 3T3 cells 69 hours after infection following 12 hours of labeling with ^3H -thymidine ($10 \mu\text{Ci/ml}$). Enzyme digests were carried out for two hours at 37° and terminated by the addition of stop buffer. Samples were then applied to composite polyacrylamide-agarose gels. Electrophoresis followed at 40 V for 12 hours. To all samples was added ^{23}P -labeled Ad 2 DNA as marker.

7. Location of the termini of replication

It is well established for the human adenoviruses that the termini of replication are located at both molecular ends of the DNA (Horwitz, 1974; Schilling et al., 1979; Tolun and Pettersson, 1975; Sussenbach and Kuijk, 1977), an observation which in connection with later determinations of the location of the origin (Horwitz, 1976; Weingärtner et al., 1976; Sussenbach and Kuijk, 1978) defines a gross mechanism of replication for adenoviruses. In an attempt to demonstrate that mouse adenovirus is a typical adenovirus in terms of function I wished to establish that the termini of replication for this virus are also at the molecular ends of the DNA, and in the process find as much out about the mechanism of replication as possible.

The approach I took in locating the termini was the same as was employed for Ad 2 (Schilling et al., 1975; Tolun and Pettersson, 1975), Ad 5 (Sussenbach and Kuijk, 1977), SV40 (Danna and Nathans, 1972), and in locating the peptide terminus of hemoglobin (Dintzis, 1961). In this case the DNA is labeled for a period of time short compared to the time required for one round of replication. Then if only the completed double-stranded molecules are analyzed, there will be more label at the termini of replication as compared to the rest of the molecule. Experimentally this is determined by comparing the distribution of label along the genome in pulse labeled molecules to that in uniformly labeled DNA.

Since I did not know the exact time required for a round of replication, I pulse labeled infected cells for 5, 15, 20, 60, and 120 minutes, isolated the completed, double-stranded molecules, treated them and uniformly labeled, completed DNA molecules with restriction enzymes, and compared the distribution of label. A summary is presented in Table 3. It is clear that there is relatively more label in the terminal fragments of the DNA isolated after the 5 minute pulse as compared to the rest of the molecule. This indicates that the termini of replication

are at both molecular ends of the DNA. Not only that but depending on the fragment size the relative activity varies between 5 and 9 with the higher specific activity found for the smaller fragment. These results are what is expected after a short pulse if replication terminates with equal probability at either end of the DNA and shows no strand preference for initiation.

Table 3. Relative incorporation of pulse label into mouse adenovirus DNA.

pulse time (min.)	enzyme	A	B	C	D	E	F	G
5	Hpa I	1.0	1.1	5.3 ⁺	2.8	1.3	9.3 ⁺	2.7
15	Eco R1	1.0	1.6 ⁺	1.8 ⁺				
20	Bam H1	1.0	1.0	1.0	1.4 ⁺	1.3 ⁺	n.d.	
60	Bam H1	1.1	1.1	1.1	1.2 ⁺	1.0 ⁺	n.d.	
120	Bam H1	1.0	1.0	1.0	1.2 ⁺	1.0 ⁺	n.d.	

⁺ terminal fragment

n.d. not determined

8. How long does one round of replication take ?

Examination of the data presented in Table 3 shows that as the pulse time becomes longer the asymmetry of the distribution of label becomes less pronounced. By 15 minutes and certainly by 20 minutes there is little if any more label in the terminal portions of the genome as compared to the rest of the molecule. This means that the time required for one round of replication is around 15 or 20 minutes which is exactly what was found for Ad 2 using the same method (Schilling et al., 1975).

9. Location of the origins of replication

Although the above data showed that the termini of replication are clearly at the molecular ends of mouse adenovirus DNA several possibilities existed for the location of the origin(s). They could be located internally in the genome or at both molecular ends of the DNA as was discussed for Ad 2 DNA replication at an equivalent stage of investigation (Schilling et al., 1975; Tolun and Pettersson, 1975; Horwitz, 1976; Weingärtner et al., 1976). For Ad 2 this was resolved by examining the distribution of label in the separated strands of pulse-labeled, completed, double-stranded DNA molecules either by hybridization analysis (Weingärtner et al., 1976) or by electrophoresis (Horwitz, 1976; Weingärtner et al., 1976). If the origin is internal in the genome there should be an equal distribution of radioactivity between the two strands but if there are two equivalent origins, one at either end of the DNA, then there should be an unequal distribution. Specifically, the label should be in the strand containing the 3' terminus. To test these expectations I analyzed the distribution of radioactivity in the separated strands of the Eco RI B and C fragments (terminal fragments) of mouse adenovirus DNA which had been labeled for 15 minutes with ^3H -thymidine. I compared this distribution to that found in DNA which was uniformly labeled also with ^3H -thymidine. A typical gel is shown in Figure 8 and a summary of the data in Table 4. There is relatively more label in the lower strand of both the B and C fragments in the case of the uniformly labeled DNA. However, in the case of pulse labeled DNA there is strikingly more label in the upper strand of both fragments. After correction for the asymmetric distribution of label in the strands of the uniformly labeled DNA there is about 5 times more label in the upper strand of both terminal fragments as compared to the lower strand of each.

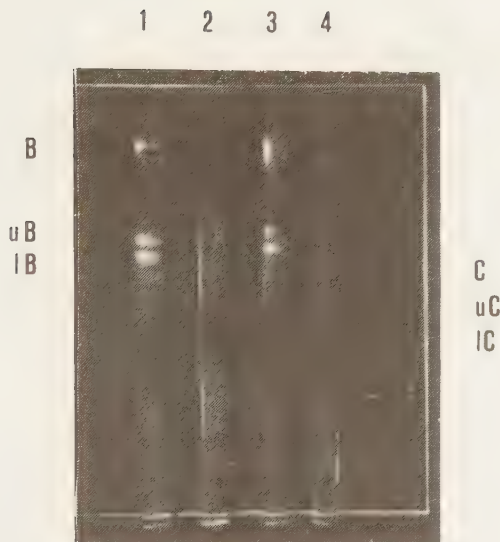


Fig.8. Separated strands of the mouse adenovirus DNA Eco R1 B and C fragments.
For gels 1 and 2 preparation of the samples and electrophoresis were carried out exactly as described in the methods section. Each sample was 12 μ g of DNA. For samples 3 and 4 each sample was also 12 μ g of DNA. However, instead of being neutralized with PET buffer the gel slices were soaked in ice-cold 0.015 M NaOH for the same length of time. B and C indicate the position of the undenatured fragments. uB and uC indicate the position of the upper strand, and lB and lC that of the lower strand.

Table 4. Distribution of pulse label in the separated strands of the molecular ends of mouse adenovirus DNA.

Fragment	uniformly labeled cpm	pulse labeled ratio	pulse labeled cpm	corrected ratio	³² P cpm
Eco R1 B		.51		4.7	
upper	586		1241		234
lower	1149		513		69
Eco R1 C		.62		5.2	
upper	870		666		
lower	1408		206		

In order to determine which strand is which with respect to the polarity of the DNA I labeled the one free 5' end of the terminal Eco RI B fragment with ^{32}P in the reaction with T4 polynucleotide kinase. This results in the strand with the 3' terminus of the DNA being labeled. This is also the strand in which pulse label is expected if replication originates at the molecular ends of the DNA. As shown in Table 4 the ^{32}P and ^3H are in the same strand. Therefore, as was found for Ad 2 and Ad 5, mouse adenovirus DNA replication originates at the 5' end and terminates at the 3' end of the DNA.

10. Replication occurs via single-stranded intermediates

The method I used to isolate the double-stranded, completed molecules after pulse labeling the DNA was chromatography on BND cellulose. Although I was primarily interested in the double-stranded forms I eluted the single-stranded material as well in order to gain insight into the role these forms may have in viral DNA replication. It is well established at least for the case of Ad 2 and Ad 5 that DNA replication proceeds via single-stranded intermediates. The existence of these forms was determined by a variety of methods. DNA isolated from infected cells was analyzed with respect to its sedimentation behaviour in neutral sucrose (Pearson and Hanawalt, 1971), its buoyant density in neutral CsCl (Pettersson, 1973), its retention on BND cellulose (Robin et al., 1973), its sensitivity to single-stranded DNA specific nuclease (Pettersson, 1973), and its physical structure as determined by electron-microscopy (Lechner and Kelly, 1977).

Shown in Table 5 is the distribution of label in the single-stranded and double-stranded material eluted from BND cellulose as a function of pulse time. For very short pulses (5 minutes) there is approximately 4-5 times as much single-stranded as double-stranded material. However, as the pulse time increases, the ratio between the two forms decreases indicating accumulation of completed DNA molecules. After one hour of labeling the amount of label in each of the two species is equal and after two hours over 2/3 of the radioactivity exists in the double-stranded form.

Table 5. Distribution of label in single-stranded and double-stranded DNA as a function of pulse time.

pulse time (minutes)	double-stranded	single-stranded	ratio
5	42,300	204,000	4.52
10	58,700	184,700	3.15
20	67,500	234,150	3.47
60	466,600	468,900	1.01
120	656,850	419,400	.64

I analyzed the nature of the single stranded material by treating it with the restriction endonuclease Eco R1 to show that it is of viral origin. The DNA which elutes as being single-stranded in the case of Ad 2 is not completely single-stranded but contains approximately 30% random double-stranded regions as well (Pettersson, 1973; Lavelle et al., 1975; Flint et al., 1976). Therefore it is possible to determine the restriction pattern. It is identical to that of the double-stranded material. This means that the decrease in single-stranded material with respect to that which is double-stranded reflects a conversion of this into completed DNA molecules and ultimately to virus particles.

11. Transfection of HeLa cells with Ad 2 DNA-protein complex

The optimum conditions for transfecting HeLa cells with Ad 2 or Ad 5 have been established by Graham and Van der Eb (1973), Frost and Williams (1978), and Galos et al. (1979). DNA-protein complex (Galos et al., 1979) is precipitated in the presence of Ca^{+2} (Graham and Van der Eb, 1973), applied to the monolayer of cells and then several hours later after the infection process has started, the cells are treated with either DMSO (Stow and Wilkie, 1976) or glycerol (Frost and Williams, 1978). Since the highest specific activities determined under these conditions seemed to occur with very small amounts of DNA and since I was interested in introducing as much DNA as possible into the cells in order to be able to measure DNA synthesis, I investigated the relationship between plaque formation and the amount of DNA used

for transfection. A summary of the data is shown in Table 6. The total amount of DNA applied in each case was kept constant by the addition of carrier DNA. Although there is some variation in the data I can say that the number of plaques formed is quite constant with 10-0.01 μ g DNA within the same experiment.

Table 6. Dependence of plaque formation on the amount of DNA.

μ g DNA	DMSO	glycerol
10	102, 242	172
1	60, 7, 9	8
.57	23, 36	-
.1	-	200
.057	51	12
.01	100, 22, 32	200, 26
.0057	-	7

Whether the cells are treated with DMSO or with glycerol seems not to make any difference. The relatively constant number of plaques regardless of the amount of DNA means that the specific infectivity (pfu/ μ g DNA) with smaller amounts of DNA is higher. However, because of this, the designation of specific infectivity is meaningless because there is not a linear relationship between the number of plaques and the amount of DNA.

One possibility for this could be that only cells in a certain condition are susceptible to transfection (Stow and Wilkie, 1976; H.Wolf, personal communication). If this were the case the number of plaques with a constant amount of DNA should be proportional to the number of cells. The summary of such an experiment is shown in Table 7. Contrary to the expectation, there appear to be fewer plaques with more cells. Clearly further investigations must be carried out.

Table 7. Dependence of plaque formation on the number of cells.

plaques/100 mm plate	plaques/60 mm plate	plaques/35 mm plate
0	1, 2, 2, 2, 3	6, 9, 11, 15, 33

Instead of measuring the infectivity of the DNA by plaque formation, I wanted to measure DNA synthesis in the manner described for the growth kinetic experiments. Ultimately, I wanted to score the replication of a replicon in this manner. Therefore, I needed transfection conditions which approximated an efficient infection. As an example only 12.5 pg DNA in the form of infectious virus are required for 3×10^5 cells to become infected and under these conditions DNA synthesis is measurable as it first begins.

In the case of transfection only about 100 cells are infected as measured by plaque formation with 10-0.01 μ g DNA. Therefore DNA synthesis does not become apparent until a very long time after infection. The data are shown in Table 8. The problem then becomes one of increasing the efficiency of transfection by a factor of at least 1000 (from 100 cells becoming infected to 1×10^5 or more cells becoming infected). However, as shown by the independence of plaque number on the amount of DNA used for transfecting the cells, it is not just a simple matter of increasing the amount of DNA applied to the cells.

Table 8. Virus production and DNA synthesis after transfection.

<u>Time (hours)</u>	<u>pfu/ml</u>	<u>DNA</u>
23.5		-
46.5		-
51		-
67		-
71	40	-
88.5		+
90.5	1.9×10^4	
111.5		+
115	1.2×10^6	
135.5		+

Perhaps the efficiency can be increased by use of DNA encapsulated in liposomes. This technique has been successfully employed to encapsulate polio virus (Wilson et al., 1977) and its RNA (Wilson et al., 1979) in order to introduce it into cells. However, preliminary experiments with Ad 2 DNA indicate that there is no drastic improvement in efficiency by this method (I.Pfitzinger and E.-L.Winnacker, unpublished observations), in fact no plaques were obtained at all.

DISCUSSION

I have studied the growth of mouse adenovirus and begun studies on the replication of the its DNA as a prerequisite to future investigations concerning the definition of the minimal adenovirus replicon and to its use as a eukaryotic vector. The results show that the attributes which appear to be common to the human adenoviruses are indeed applicable to another species. This provides generality for the criteria which are believed to distinguish the adenoviruses as a special class by themselves.

Mouse adenovirus grows well enough on 3T3 cells to obtain enough virus for studies leading to characterization of its physical properties as well as to carry out functional and biochemical investigations. The DNA has a molecular weight of $19-20 \times 10^6$ daltons. Incorporated into this length of nucleic acid are several features typical of adenoviruses. There is evidence for a protein bound, probably covalently, to the 5' end of the DNA. The DNA also exhibits an inverted terminal repeat (Larsen and Nathans, 1977; Antoine, 1979) as is found for other adenoviruses (Garon et al., 1972; Wolfson and Dressler, 1972; Steenbergh et al., 1977; Arrand and Roberts, 1979; Tolun et al., 1979). Later I discuss several structural features of this repeat which has been sequenced (Antoine, 1979). Further, I compare the sequence to the sequences of the terminal repetitions of several other adenoviruses (Tolun et al., 1979) as well as to the origin region of the mouse papova virus, polyoma (Soeda et al., 1980). I also compare the human adenovirus terminal repeat sequences to the origin region of the human papova virus, BKV (Seif et al., 1979), and the terminal repetition of simian adenovirus type 7 to the origin of SV40 (Subramanian and Shenk, 1978). Because these sets of viruses have common hosts, a common sequence may reflect some host specific signal which is relevant for cellular nucleic acid metabolism, especially DNA replication.

Larsen and Nathans (1977) on the basis of buoyant density measurements, and H.Delius (personal communication) by partial denaturation of the DNA have found that mouse adenovirus DNA contains 56-70% AT residues. In comparison to all of the human adenoviruses and to simian adenovirus type 7 this is extremely AT rich. Group A of the human adenoviruses contains 48-49% AT, group B 49-52%, and group C 55-60% (Pina and Green, 1965). For the human adenoviruses there is a relationship between the oncogenicity and the GC content: the higher the GC content, the less oncogenic the virus (Pina and Green, 1965). In this context mouse adenovirus would be extremely oncogenic, something to keep in mind if it is ever exploited as a carrier of genetic material. However, simian adenovirus type 7 with a GC content of 60% is extremely oncogenic (Burnett and Harrington, 1968). Therefore, this relationship found for the human adenoviruses is perhaps not applicable to viruses of other species. Furthermore, there are no reports of the ability of mouse adenovirus to either induce tumors in appropriate hosts or to transform cells in tissue culture. This does not mean however, that these investigations should not be carried out.

The distribution of the AT residues in mouse adenovirus DNA (H.Delius, personal communication) is asymmetric. On this basis the physical map of mouse adenovirus DNA may be oriented with the AT rich portion to the right, according to one of the criteria recently adopted for the orientation of adenovirus genomes (Adenovirus Strand Nomenclature: A Proposal. *J.Virol.* 22, 830-831 (1977)). This orientation, according to accepted conventions leads to an alignment of the restriction maps identical to that originally proposed by Larsen and Nathans (1977) but opposite to that recently proposed by Larsen et al. (1979). Larsen et al. (1979) changed the orientation when they attempted to align the physical map of mouse adenovirus to correspond to that of human adenovirus type 2. They defined the end of mouse adenovirus DNA most often found in defective particles as being the left-hand end in analogy

to what Tibbits (1977) found for human adenovirus type 3. They also attempted to align the regions of homology found between mouse adenovirus DNA and human adenovirus type 2 DNA. This homology occurs presumably in the region coding for the hexon gene of both viruses, which is not unexpected since it is the hexon protein which provides the general adenovirus antigen which defines an adenovirus of any species as being an adenovirus (Schlesinger, 1969). Unfortunately neither of these criteria are included in the proposal and agreement quoted above. Resolution of this question will depend on the answer obtained by determination of transcription maps for mouse adenovirus.

Do the functional features of mouse adenovirus DNA and mouse adenovirus itself agree as well as the structural features to what has been observed for the other adenovirus, specifically the human types 2 and 5 ?

One difference is the yield of virus. Unfortunately there are numerous steps and phases of growth between the initial interaction of the virus with its host cell and the ultimate appearance of mature virus particles. Some of these have been genetically defined for the human adenoviruses (Ginsberg and Young, 1977). The procedure employed in isolating the virus, as well as the assay used to measure the yield may also have an influence. I have studied only one small portion of the growth cycle, DNA replication. This particular process in mouse adenovirus appears not to differ either kinetically or mechanistically to that of Ad 2. It is not likely therefore to be the cause of the difference in virus yield.

Another difference is the time after a normal infection with a high multiplicity of infection that viral DNA synthesis begins. For mouse adenovirus this is 36 hours, for Ad 2 and Ad 5 it is 7-8 hours (Green et al., 1970), and for Ad 12 it is 9-10 hours (Green et al., 1970). Again the extreme difference observed with mouse adenovirus is not caused by the actual synthesis of DNA which appears to occur at the same rate as for Ad 2 (Schilling

et al., 1975). One of the early functions (processes occurring before the onset of DNA synthesis) probably determines the length of this lag time. It is perhaps interesting to note that the highly oncogenic Ad 12 with DNA with a high AT content has a longer early phase than Ad 2, Ad 5 or Ad 7 (Green et al., 1970). Mouse adenovirus has an even longer one and a higher AT content. Also, infection of nonpermissive cells with almost any adenovirus produces transformed cells. In this case DNA synthesis never occurs which means that the early phase is very long indeed. Perhaps there is a relationship between the rapidity of the start of DNA synthesis and the oncogenicity of the virus.

The process of DNA synthesis in mouse adenovirus appears to be identical to that in Ad 2 even though it begins at a longer time after infection. The length of time required for one round of replication, 20 minutes, is exactly the same as that determined for Ad 2 (Schilling et al., 1975). The scheme that both of these viruses employ appears to be identical. For both viruses replication originates and terminates at both molecular ends of the DNA. This I determined for mouse adenovirus by first analyzing the distribution of radioactivity in DNA which had been pulse labeled for short periods of time compared to that required for one round of replication. I found that there is an increasing gradient of radioactivity extending from the internal portions of the DNA towards the molecular ends as was determined for Ad 2 in the same manner (Schilling et al., 1975). Knowing this, which shows that the termini are at these terminal positions, I proceeded to analyze the distribution of label in each of the strands of the ends of the DNA, in order to determine if the origin has an internal or external position. The asymmetric distribution clearly indicates that the origin too is at the molecular ends of the DNA. Further identification of the orientation of the strands by uniquely labeling one of them showed that the direction of replication is consistent with a terminal origin as well.

These determinations provide necessary information for experiments designed to define the regions of the DNA required for replication. The approach I would take is to delete regions of the DNA and then determine if the remaining truncated molecule can still replicate in the presence of the appropriate helper functions. Since the origins and termini are at the or near the molecular ends of the DNA I would start by deleting internal portions of the genome.

The technology for this "genetic engineering" is already available. There is precedent for these deleted molecules being able to replicate as well since they are perhaps similar to the naturally occurring defective particles of Ad 2 (Daniell, 1976), Ad 3 (Tibbits, 1977), and mouse adenovirus (Larsen and Nathans, 1977). Somehow these are formed, although the mechanism may not involve actual replication of the truncated DNA. They could also be formed by a mechanism involving processing of full length molecules. With some viruses such as VSV the defective particles seem to replicate very efficiently to such an extent that they ultimately outgrow the wild type virus (Huang, 1973).

In the worst case besides not replicating at all each of the constructed molecules would undergo only one round of replication, if that. What the behaviour of the deleted molecules will be remains to be seen. However, in the case of the worst case, it is important to be able to observe the first round of DNA replication in order to have the best chance of being able to detect any synthesis at all.

Since these molecules are naked DNA and not virus particles a normal route of infection cannot be followed in order to introduce the DNA into the cells. Unfortunately transfection is a very inefficient process in which only 40-200 cells score as having been infected by forming a plaque. This number is independent of the amount of DNA so simply using more DNA does not result in more cells becoming infected. In spite of the inefficiency, transfection of HeLa cells with Ad 2 DNA

does lead to virus production as shown in Table 8. This means that the process does work, although not well enough to be useful as an assay for a replicon yet. Obviously there are several different approaches to improving the efficiency involving both the DNA and the cells. The influence of these two components is worth investigating in its own right and interest.

What general information have these studies provided concerning adenovirus replication ?

Because mouse adenovirus is a species distinct from the well studied human adenoviruses but yet carries out replication in a similar if not identical fashion, the mechanism may be considered general if not universal for adenoviruses. Further, generality may be obtained by comparing the primary sequence of the terminal repetition, believed to contain the origin of replication (Sussenbach and Kuijk, 1978; Winnacker, 1978), of several adenoviruses to each other (Tolun et al., 1979) and to the origin regions of unrelated viruses, the papova viruses for instance, which infect the same host cell. Since both of these viruses use host enzymes for replication, similarities in sequence may indicate universal signals for the enzymes. Fortunately, such a comparison is possible because the sequence of the terminal repeats of several adenoviruses has been determined (Antoine, 1979; Tolun et al., 1979) as has the sequence of the genomes of the papova viruses, BKV (Seif et al., 1979), SV40 (Fiers et al., 1978; Reddy et al., 1978) and polyoma (Soeda et al., 1980). In addition Subramanian and Shenk (1978) have defined the origin of SV40 DNA replication.

Most striking as seen in Figure 9 is that the terminal repeats of all of the adenoviruses so far sequenced show homology regardless of species. Ad 2 and Ad 5 are identical (Tolun et al., 1979) and the others share specific regions of homology. Mouse adenovirus DNA does not have these regions but does have, beginning at each DNA terminus, 17 base pairs which are identical to those occurring in Ad 2 and Ad 5 DNA at the same positions. Part of the reason that the internal regions are missing is

that mouse adenovirus DNA is extremely AT rich. The homologies involve mostly GC residues. Also there is really no GC rich region following the terminal AT rich region of mouse adenovirus DNA as is found in the terminal repetition of other adenoviruses.

If there is a universal origin sequence for adenovirus, the most likely candidate in terms of the greatest degree of homology among the adenoviruses themselves would be the base pairs in positions 9-17 in the mouse adenovirus (Ad 2, Ad 5) genome. These 9 base pairs, 5'-ATAATATAC-3', are common to all of the terminal repeats for which a sequence is known. However, the structure of this AT rich sequence is more like a promotor (Hogness box) or RNA processing signal (Soeda et al., 1980). On the other hand mouse adenovirus DNA is reportedly replicated in vitro by extracts from cells infected with Ad 2 (Th.Reiter and E.-L.Winnacker, unpublished observations), which is an indication that these terminal regions of homology are important since they are the most extensive sequence common to both viruses.

Comparison of the adenovirus terminal repeats with known origin sequences of the papova viruses of homologous host type, BKV in the case of the human adenoviruses, SV40 in the case of SA 7, and polyoma and the case of mouse adenovirus, reveals another potential origin region. This is in all cases at an internal position of the terminal repeat of the adenovirus DNA where there are several base pairs which are homologous to sequences known to be in the origin region of the papova viruses. This is shown in Figure 9. Because these papova virus sequences are origin sequences and because the viruses infect identical hosts, these homologies most likely represent replication signals which are possibly relevant for host cell DNA replication.

Tolun et al. (1979) found regions of homology between the various adenovirus terminal repeats and a region of SV40 which is located near the origin. These sequences are, however, not directly at the origin but rather occur between the origin and

the 5' end of the long leader sequence for the late mRNA species (Fiers et al., 1978; Reddy et al., 1978) and within a region which can be deleted (Shenk, 1977). In any case a subset of this sequence which has the structure 5'-GGGXGGAG-3' is found at the origin in BKV (Seif et al., 1979). In this virus it follows a strikingly AT rich region 17 base pairs long. This is its relative position in the human adenoviruses types 2, 5 and 12, where it occurs after the AT rich region of the terminal repeat. A subset of the sequence, 5'-GGCGG-3', occurs in an equivalent position in SA 7 and is included in the origin of SV40 (Subramanian and Shenk, 1978). In mouse adenovirus DNA there are 7 base pairs, 5'-TGGCCCG-3', which occur also in the origin region of polyoma (Soeda et al., 1980). The structure of this sequence is different than the homology discussed above but it too occurs in approximately the same position.

Exactly what these sequences are is unclear. They are perhaps recognition or binding signals for some enzyme crucial to replication. It is known for example that the SV40 large T antigen binds at the origin and is required for the initiation of replication (Tjian, 1978).

In order to answer these questions functional studies will have to be carried out.

REFERENCES

- Allet, B., Jeppeson, P.G.N., Katagiri, K.J., and Delius, H. (1973). Mapping the DNA fragments produced by cleavage of lambda DNA with endonuclease RI. *Nature* 241, 120-123.
- Anderson, T.F. (1948). The activation of the bacterial virus T4 by L-tryptophan. *J.Bact.* 55, 637-649.
- Antoine, G. (1979). Primärstruktur der molekularen Enden von Maus Adenovirus DNA. Diplomarbeit, Universität München.
- Arens, M., Yamashita, T., Padmanabhan, R., Tsuruo, T., and Green, M. (1977). Adenovirus deoxyribonucleic acid replication, characterization of the enzyme activities of a soluble replication system. *J.Biol.Chem.* 252, 7949-7954.
- Arrand, J.R., and Roberts, R.J. (1979). The nucleotide sequences at the termini of adenovirus-2 DNA. *J.Mol.Biol.* 128, 577-594.
- Backmann, K., Betlach, M., Boyer, H.W., and Yanofsky, S. (1978). Genetic and physical studies on the replication of Col E1-type plasmids. *Cold Spring Harbor Symposia on Quantitative Biology* 43, 69-76.
- Bellett, A.J.D., and Younghusband, H.B. (1972). Replication of the DNA of chick embryo lethal orphan virus. *J.Mol.Biol.* 72, 691-709.
- Carusi, E.A. (1977). Evidence for blocked 5'-termini in human adenovirus DNA. *Virology* 76, 380-394.
- Cavalier-Smith, T. (1974). Palindromic base sequences and replication of eukaryotic chromosome ends. *Nature* 250, 467-470.

Daniell, E. (1976). Genome structure of incomplete particles of adenovirus. *J.Virol.* 19, 685-708.

Danna, K.J., and Nathans, D. (1972). Bidirectional replication of simian virus 40 DNA. *Proc.Nat.Acad.Sci. USA* 69, 3097-3100.

Davidson, N., and Szybalski, W. (1971). Physical and chemical characterization of lambda DNA. In: *The Bacteriophage Lambda* (Hershey, A.D., ed.), Cold Spring Harbor Laboratory, pp.45-82.

Dintzis, H.M. (1961). Assembly of the peptide chains of hemoglobin. *Proc.Nat.Acad.Sci. USA* 47, 247-261.

Doerfler, W., and Kleinschmidt, A.;. (1970). Denaturation pattern of the DNA of adenovirus type 2 as determined by electron microscopy. *J.Mol.Biol.* 50, 579-593.

Enders, J.F., Bell, J.A., Dingle, J.H., Thomas, F.jr., Hillman, M.R., Huebner, R.J., and Payne, A.M.-M. (1956). Adenoviruses: Group name proposed for new respiratory-tract viruses. *Sci.* 124, 119-120.

Fangman, W.L. (1978). Separation of very large DNA molecules by gel electrophoresis. *Nuc.Acids.Res.* 5, 653-669.

Fiers, W., Contreras, R., Haegeman, G., Rogie, R., Van de Voorde, A., Van Heuverswyn, H., Van Herreweghe, J., Volekaert, G., and Ysebaert, M. (1978). Complete nucleotide sequence of SV40 DNA. *Nature* 273, 113-120.

Flint, J. (1977). The topography and transcription of the adenovirus genome. *Cell* 10, 153-166.

Flint, S.J., Berget, S.M., and Sharp, P.A. (1976). Characterization of single-stranded viral DNA sequences present during replication of adenovirus types 2 and 5. *Cell* 9, 559-571.

Friend, C. (1957). Cell-free transmission in adult swiss mice of a disease having the character of a leukemia. J.Exp. Med. 105, 307-318.

Frost, E., and Williams, J. (1978). Mapping temperature-sensitive and host-range mutants of adenovirus type 5 by marker rescue. Virology 91, 39-50.

Fütterer, J. (1978). DNA-Protein-Komplexe in Adenovirus-infizierten HeLa-Zellen. Diplomarbeit, Universität München.

Galos, R.S., Williams, J., Binger, M.-H., and Flint, J.S. (1979). Location of additional early gene sequences in the adenovirus chromosome. Cell 17, 945-956.

Garon, C.F., Berry, K.W., and Rose, J.A. (1972). A unique form of terminal redundancy in adenovirus DNA molecules. Proc.Nat.Acad.Sci. USA 69, 2391-2395.

Gilbert, W., and Muller-Hill, B. (1967). The lac operator is DNA. Proc.Nat.Acad.Sci. USA 58, 2415-2421.

Ginsberg, H.S., and Young, C.S.H. (1977). Genetics of adenoviruses. Comprehensive Virology 9, 27-88.

Graham, F.L., and Van der Eb, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456-467.

Green, M. (1970). Oncogenic viruses. Ann.Rev.Biochem. 39, 701-756.

Green, M., Pina, M., Kimes, R., Wensink, P.C., MacHattie, L.A., and Thomas, C.A. jr. (1967). Adenovirus DNA I. Molecular weight and conformation. Proc.Nat.Acad.Sci. USA 57, 1302-1309.

Grütter, M., and Franklin, R.M. (1974). Studies on the molecular weight of the adenovirus type 2 hexon and its subunit. *J.Mol.Biol.* 89, 163-178.

Hartley, J.W., and Rowe, W.P. (1960). A new mouse virus apparently related to the adenovirus group. *Virology* 11, 645-647.

Hashimoto, K., Sugiyama, T., and Sasaki, S. (1966). An adenovirus isolated from the feces of mice I. Isolation and identification. *Japan.J.Microbiol.* 10, 115-125.

Heck, F.C. jr., Sheldon, W.G., and Gleiser, C.A. (1972). Pathogenesis of experimentally produced mouse adenovirus infection in mice. *Am.J.Vet.Res.* 33, 841-846.

Hillemann, M.R., and Werner, J.H. (1954). Recovery of new agent from patients with acute respiratory illness. *Proc. Soc.Exp.Biol.* 85, 183-185.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J.Mol.Biol.* 26, 365-369.

Hobom, G., Grosschiedl, R., Lusky, M., Scherer, G., Schwarz, E., and Kössel, H. (1978). Functional analysis of the replicator structure of lambdoid bacteriophage DNAs. *Cold Spring Harbor Symposia on Quantitative Biology* 43, 169-178.

Horwitz, M. (1974). Location of the origin of DNA replication in adenovirus type 2. *J.Virol.* 13, 1046-1054.

Horwitz, M. (1976). Bidirectional replication of adenovirus type 2 DNA. *J.Virol.* 18, 307-315.

Huang, A.S. (1973). Defective interfering virus. *Ann.Rev. Microbiol.* 27, 101-117.

Huebner, R.J. (1967). Adenovirus-directed tumor and T antigens. Perspectives in Virology 5, 147-166.

Jacob, F., Brenner, S., and Cuzin, F. (1963). On the regulation of DNA replication in bacteria. Cold Spring Harbor Symposia on Quantitative Biology 28, 329-348.

Kathmann, P., Schick, J., Winnacker, E.-L., and Doerfler, W. (1976). Isolation and characterization of temperature-sensitive mutants of adenovirus type 2. J.Virol. 19, 43-53.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Larsen, S.H., Margolskee, R.F., and Nathans, D. (1979). Alignment of the restriction map of mouse adenovirus FL with that of human adenovirus 2. Virology 97, 406-414.

Larsen, S.H., and Nathans, D. (1977). Mouse adenovirus: Growth of plaque-purified FL virus in cell lines and characterization of viral DNA. Virology 82, 182-195.

Lavelle, G., Patch, C., Khoury, G., and Rose, J. (1975). Isolation and partial characterization of single-stranded adenoviral DNA produced during synthesis of adenovirus type 2 DNA. J.Virol. 16, 775-782.

Lechner, R.L., and Kelly, T.J. jr. (1977). The structure of replicating adenovirus 2 DNA molecules. Cell 12, 1007-1020.

Luria, S.E., and Steiner, D.L. (1954). The role of calcium in the penetration of bacteriophage T5 into its host. J.Bact. 67, 635-639.

MacHattie, L.A., Richie, D.A., Thomas, C.A. jr., and Richardson, C.C. (1967). Terminal repetition in permuted T 2 bacteriophage DNA molecules. *J.Mol.Biol.* 23, 355-363.

Maizel, J.V. jr., White, D.O., and Scharff, M.D. (1968). The polypeptides of adenovirus I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36, 115-125.

Maxam, A.M., and Gilbert, W. (1977). A new method for sequencing DNA. *Proc.Nat.Acad.Sci. USA* 74, 560-564.

Moore, D.D., Denniston-Thompson, K., Kruger, K.E., Furth, M.E., Williams, B.G., Daniels, D.L., and Blattner, F.R. (1978). Dissection and comparative anatomy of the origins of replication of lambdoid phages. Cold Spring Harbor Symposia on Quantitative Biology 43, 155-163.

Murray, R.E., and Green, M. (1973). Adenovirus DNA IV. Topology of adenovirus genomes. *J.Mol.Biol.* 74, 735-738.

Nicolson, M.O., and McAllister, R.M. (1972). Infectivity of human adenovirus-1 DNA. *Virology* 48, 14-21.

Pearson, G.D., and Hanawalt, P.C. (1971). Isolation of DNA replication complexes from uninfected and adenovirus infected cells. *J.Mol.Biol.* 62, 65-80.

Perlman, D., and Huberman, J.A. (1977). Preparation of large quantities of separated strands from simian virus 40 DNA restriction fragments by low-temperature low-salt agarose gel electrophoresis. *Anal.Biochem.* 83, 666-677.

Pettersson, U. (1973). Some unusual properties of replicating adenovirus type 2 DNA. *J.Mol.Biol.* 81, 521-527.

Pettersson, U., Mulder, C., Delius, H., and Sharp, P.A. (1973). Cleavage of adenovirus type 2 DNA into six unique fragments by endonuclease R.R1. Proc.Nat.Acad.Sci. USA 70, 200-204.

Philipson, L., and Lindberg, U. (1974). Reproduction of adenoviruses. Comprehensive Virology 3, 143-227.

Pina, M., and Green, M. (1965). Biochemical studies on adenovirus multiplication. IX. Chemical and base composition analysis of 28 human adenoviruses. Proc.Nat.Acad.Sci. USA 54, 547-551.

Pina, M., and Green, M. (1969). Biochemical studies on adenovirus multiplication. XIV. Macromolecule and enzyme synthesis in cells replicating oncogenic and nononcogenic human adenovirus. Virology 36, 573-586.

Pribnow, D. (1975). Nucleotide sequence of an RNA polymerase binding site at an early T7 promotor. Proc.Nat.Acad.Sci. USA 72, 784-788.

Puck, T.T. (1949). A reversible transformation of T1 bacteriophage. J.Bact. 57, 647-655.

Reddy, V.B., Thimmappaya, B., Dhar, R., Subramanian, K.W., Zain, B.S., Pan, J., Ghosh, P.K., Celma, M.L., and Weissman, S.M. (1978). The genome of simian virus 40. Science 200, 494-502.

Rekosh, D.M.K., Russell, W.C., Bellett, A.J.D., and Robinson, A.J. (1977). Identification of a protein linked to the ends of adenovirus DNA. Cell 11, 283-295.

Reznikoff, W.S., Miller, J.H., Scaite, J.G., and Beckwith, J.R. (1969). A mechanism for repressor action. J.Mol.Biol. 43, 201-213.

Richardson, C.C. (1965). Phosphorylation of nucleic acid by an enzyme from T4 bacteriophage-infected *Escherichia coli*. *Proc.Nat.Acad.Sci. USA* 54, 158-165.

Ritchie, D.A., Thomas, C.A.jr., MacHattie, L.A., and Wensink, P.C. (1967). Terminal repetition in non-permuted T3 and T7 bacteriophage DNA molecules. *J.Mol.Biol.* 23, 365-376.

Robin, J., Bourgaux-Ramoisy, D., and Bourgaux, P. (1973). Single-stranded regions in replicating DNA of adenovirus type 2. *J.Gen.Virol.* 20, 233-237.

Robinson, A.J., and Bellett, A.J.D. (1974). A circular DNA protein complex from adenoviruses and its possible role in DNA replication. *Cold Spring Harbor Symposia on Quantitative Biology* 39, 523-531.

Rouse, H.C., Bonifas, V.H., and Schlesinger, R.W. (1963). Dependence of adenovirus replication on arginine and inhibition of plaque formation by pleuropneumonia-like organisms. *Virology* 20, 357-365.

Rowe, W.P., Hartley, J.W., and Huebner, R.J. (1958). Serotype composition of the adenovirus group. *Proc.Soc.Exp.Biol. Med.* 97, 465-470.

Rowe, W.P., Huebner, R.J., Gilmore, L.K., Parrott, R.H., and Ward, T.G. (1953). Isolation of a cytopathic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc.Soc.Exp.Biol.Med.* 84, 570-573.

Russell, W.C., and Becker, Y. (1968). A maturation factor for adenovirus. *Virology* 35, 18-27.

Schaller, H., Gray, C., and Herrmann, K. (1975). Nucleotide sequence of an RNA polymerase binding site from the DNA of bacteriophage fd. *Proc.Nat.Acad.Sci. USA* 72, 737-741.

Schick, J., Baczko, K., Fanning, E., Groneberg, J., Burger, H., and Doerfler, W. (1976). Intracellular forms of adenovirus DNA: Integrated form of adenovirus DNA appears early in productive infection. Proc.Nat.Acad.Sci. USA 73, 1043-1047.

Schilling, R., Weingärtner, B., and Winnacker, E.-L. (1975). Adenovirus type 2 DNA replication II. Termini of DNA replication. J.Virol. 16, 767-774.

Schlesinger, R.W. (1969). Adenoviruses: The nature of the virion and of controlling factors in productive or abortive infection and tumorigenesis. Adv.Virus Res. 14, 1-61.

Seif, I., Khoury, G., and Dhar, R. (1979). The genome of human papovavirus BKV. Cell 18, 963-977.

Shenk, T. (1977). A biochemical method for increasing the size of deletion mutations in simian virus 40 DNA. J.Mol.Biol. 113, 503-515.

Shenk, T. (1978). Construction of a viable SV40 variant containing two functional origins of DNA replication. Cell 13, 791-798.

Soeda, E., Arrand, J.R., Smolar, N., Walsh, J.E., and Griffin, B.E. (1980). Coding potential and regulatory signals of the polyoma virus genome. Nature 283, 445-453.

Steenbergh, P.H., Maat, J., van Ormondt, H., and Sussenbach, J.S. (1977). The nucleotide sequence at the termini of adenovirus type 5 DNA. Nuc.Acid.Res. 4, 4371-4389.

Stow, N.D., and Wilkie, N.M. (1976). An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. J.Gen.Virol. 33, 447-458.

Strack, H.B., and Kaiser, A.D. (1965). On the structure of the ends of lambda DNA. J.Mol.Biol. 12, 36-49.

Subramanian, K.N., Dhar, R., and Weissman, S.M. (1977). Nucleotide sequence of a fragment of SV40 DNA that contains the origin of DNA replication and specifies the 5' of "Early and Late" viral RNA I. Mapping of the restriction endonuclease sites within the Eco RII-G fragment and strategy employed for its sequence analysis. J.Biol.Chem. 252, 333-339.

Subramanian, K.N., and Shenk, T. (1978). Definition of the boundries of the origin of DNA replication in simian virus 40. Nuc.Acid.Res. 5, 3635-3642.

Sugiyama, T., Hashimoto, K., and Sasaki, S. (1967). An adenovirus isolated from the feces of mice II. Experimental infection. Japan.J.Microbiol. 11, 33-42.

Sussenbach, J.S., and Kuijk, M.G. (1977). Studies on the mechanism of replication of adenovirus DNA V. The location of termini of replication. Virology 77, 149-157.

Sussenbach, J.S., and Kuijk, M.G. (1978). The mechanism of replication of adenovirus DNA VI. Localization of the origins of the displacement synthesis. Virology 84, 509-517.

Taylor, D.P., and Cohen, S.N. (1979). Structural and functional analysis of cloned DNA segments containing replication and incompatibility regions of a miniplasmid derived from a copy number mutant of NR1. J.Bact. 137, 92-104.

Tibbits, C. (1977). Viral DNA sequences from incomplete particles of human adenovirus type 7. Cell 12, 243-249.

Tjian, R. (1978). Protein-DNA interactions at the origin of simian virus 40 DNA replication. Cold Spring Harbor Symposia on Quantitative Biology 43, 655-662.

Tolun, A., Aleström, P., and Pettersson, U. (1979). Sequence analysis of adenovirus DNA V. Nucleotide sequence of inverted terminal repetitions from different adenoviruses: Demonstration of conserved sequences and homology between simian adenovirus 7 termini and SV40 DNA. Cell 17, 705-713.

Tolun, A., and Pettersson, U. (1975). Termination sites for adenovirus type 2 DNA replication. J.Virol. 16, 759-766.

Ullrich, K. (1978). Recombinationsprozesse zwischen Virus-DNS und Restriktionsfragmenten in Adenovirus-infizierten HeLa-Zellen. Diplomarbeit, Universität München.

Van der Eb, A.J., Van Kersteren, L.W., and Van Bruggen, E.F.J. (1969). Structural properties of adenovirus DNAs. Biochim. Biophys. Acta 182, 530-541.

Van der Veen, J., and Mes, A. (1974). Serological classification of two mouse adenoviruses. Archiv f.ges.Virusforsch. 45, 386-387.

Walters, R.A., and Hildebrand, C.E. (1975). A procedure for the rapid lysis of mammalian cells prior to alkaline sucrose gradient centrifugation. Biochim.Biophys.Acta 407, 120-124.

Watson, J.D., (1972). Origin of concatemeric T7 DNA. Nat.New Biol. 239, 197-201

Weingärtner, B.H. (1977). Der Mechanismus der Adenovirus Typ 2 DNA-Replikation. Dissertation, Universität zu Köln.

Weingärtner, B., Winnacker, E.-L., Tolun, A., and Pettersson, U. (1976). Two complementary strand specific termination sites for adenovirus DNA replication. Cell 9, 259-268.

- Weinmann, R., Raskas, H.J., and Roeder, R.G. (1974). Role of DNA-dependent RNA polymerases II and III in transcription of the adenovirus genome late in productive infection. *Proc.Nat.Acad.Sci. USA* 71, 3426-3430.
- Weissbach, A. (1977). Eukaryotic DNA polymerases. *Ann.Rev. Biochem.* 46, 25-47.
- Wigand, R., Gelderblom, H., and Özel, M. (1977). Biological and biophysical characteristics of mouse adenovirus strain FL. *Arch.Virol.* 54, 131-142.
- Wilson, T., Papahadjopoulos, D., and Taber, R. (1977). Biological properties of poliovirus encapsulated in lipid vesicles: Antibody resistance and infectivity in virus-resistant cells. *Proc.Nat.Acad.Sci. USA* 74, 3471-3475.
- Wilson, T., Papahadjopoulos, D., and Taber, R. (1979). The introduction of poliovirus RNA into cells via lipid vesicles (liposomes). *Cell* 17, 77-84.
- Winnacker, E.-L. (1975). Adenovirus type 2 DNA replication I. Evidence for discontinuous DNA replication. *J.Virol.* 15, 744-758.
- Winnacker, E.-L. (1978). Adenovirus DNA: Structure and function of a novel replicon. *Cell* 14, 761-773.
- Wold, W.S.M., Green, M., and Büttner, W. (1978). Adenoviruses. In: *The Molecular Biology of Viruses* (Mayak, D.P., ed.), Marcel Dekker, Inc., New York, pp. 673-768.
- Wolfson, J., and Dressler, D. (1972). Adenovirus-2 DNA contains an inverted terminal repetition. *Proc.Nat.Acad.Sci. USA* 69, 3054-3057.

Young, E.T., and Sinsheimer, R.L. (1967). Vegetative bacteriophage lambda DNA II. Physical characterization and replication. J.Mol.Biol. 30, 165-200.

SUMMARY

Mouse adenovirus DNA has a molecular weight of $19-20 \times 10^6$ daltons. It contains 56-70% AT residues. Like the DNA of other adenoviruses, mouse adenovirus DNA has an inverted terminal repeat and a protein bound covalently to the 5' termini.

After infection of permissive 3T3 cells with mouse adenovirus DNA synthesis becomes measurable after 36 hours. This is 3-4 times longer than observed for the human adenoviruses. The mechanism of DNA replication is the same as that of Ad 2 and Ad 5. The termini and origins of replication are located at the ends of the DNA. Replication involves single-stranded intermediates and one round requires 20 minutes. This is exactly the same as found for Ad 2.

The terminal repeat of mouse adenovirus DNA has 9 base pairs which are also present in the terminal repetitions of all adenovirus DNAs for which a sequence is known. This sequence, which occurs at the very ends of the DNA, is one possibility for an origin sequence. Another possible origin sequence occurs internally in the terminal repeat. These sequences are not identical for all adenoviruses. Rather, the sequence in mouse adenovirus DNA is found at the origin of the DNA of the mouse papova virus polyoma, that of the human adenoviruses is found at the origin of the DNA of the human papova virus BKV, and that of simian adenovirus 7 at the origin of SV40 DNA.

ABBREVIATIONS

Ad X	adenovirus serotype X
BND cellulose	benzoylated, naphtoylated DEAE-cellulose
cc	cubic centimeter
cpm	counts per minute
DMSO	dimethylsulfoxide
DNA-protein complex	adenovirus DNA prepared with the terminal protein still intact and covalently bound to the 5' end
form II SV40 DNA	SV40 DNA with one nick in one strand
g	gram
M	molar
mg	milligram
ml	milliliter
mM	millimolar
mm	millimeter
m.o.i.	multiplicity of infection (pfu/cell)
mRNA	messenger RNA
μCi	microcurie
μg	microgram
nm	nanometer
pfu	plaque forming unit(s)
pg	picogram
p.i.	post infection
rpm	revolutions per minute
VSV	vesicular stomatitis virus
w/v	weight per volume

ZUSAMMENFASSUNG

Die Maus-Adenovirus-DNA hat ein Molekulargewicht von $19\text{-}20 \times 10^6$ Daltons. Der Anteil an AT-Resten beträgt 56-70 Prozent. Wie die DNA anderer Adenoviren, hat die Maus-Adenovirus-DNA eine invertierte terminale Repetition und ein Protein, das kovalent an die 5'-Enden des DNA-Moleküls gebunden scheint.

36 Stunden nach Infektion permissiver 3T3-Zellen mit Maus-Adenovirus tritt eine meßbare DNA-Synthese auf. Der Replikationsmechanismus ist wahrscheinlich der gleiche, wie er für Ad 2 und Ad 5 gefunden wurde. Als Ursprungs- und Endpunkte der DNA-Synthese dienen wohl auch bei Maus-Adenovirus die Enden des DNA-Moleküls, ebenso sind einzelsträngige Formen als Zwischenprodukte erfaßbar.

Neun Basenpaare aus dem Endbereich der terminalen Repetition von Maus-Adenovirus finden sich auch bei allen anderen daraufhin untersuchten Adenoviren. Es ist denkbar, daß diese Sequenz ganz am Ende des DNA-Moleküls als Ursprungspunkt der DNA-Replikation dient. Eine weitere Sequenz, die diesen Anspruch erfüllen könnte, liegt mitten in der terminalen Repetition und ist bei allen Adenoviren unterschiedlich. Man findet aber diese Sequenz der Maus-Adenovirus-DNA am Ursprungspunkt von Maus-Papovavirus (Polyoma), die entsprechende Sequenz der menschlichen Adenoviren am Ursprungspunkt des menschlichen Papovavirus (BKV) und die des Affen-Adenovirus Typ 7 am Ursprungspunkt von SV40.

LEBENS LAUF

Name: Margaret Pauline Temple

Geburtsdatum: 21.1.1952

Geburtsort: Midland, Michigan, USA

Eltern: Robert George Temple, Chemiker und Schreiner

Janet Caroline Temple, geb. Lang

1957 - 1962 Walter Hays Elementary School in Palo Alto, California

1963 - 1964 Chestnut Hill Elementary School in Midland, Michigan

1964 - 1965 Northeast Intermediate School in Midland, Michigan

1965 - 1967 Jordan Junior High School in Palo Alto, California

1967 - 1970 Palo Alto Senior High School in Palo Alto, California

Juni 1970 Graduation

1970 - 1973 Undergraduate Studies der Chemie an der
University of California, Santa Barbara, California

Juni 1973 Bachelor of Arts Degree with a Major in Chemistry

1973 - 1976 Graduate Studies der Biochemie unter Anleitung
von Dr. Chamberlin, Department of Biochemistry der
University of California Berkeley, California

Juni 1976 Master of Arts Degree in Biochemistry

Feb. 1976 -

Mai 1977 Arbeit bei Dr. Jovin am Max-Planck-Institut für
biophysikalische Chemie, Göttingen

Juli 1977 -

Juli 1980 Doktorarbeit unter Anleitung von Prof. Dr. E.-L. Winnacker
am Institut für Biochemie der Ludwig-Maximilians-
Universität München

